

THE ROLE OF THE HOCKEY BALL AND NEST CHAMBER
IN LOGCREEPER TURTLE (*Chelonia mydas*) EGG INCUBATION

By

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by

Raymond B. Cutler

This work is dedicated to my mother Theodora, my father Ernest, my sister
Noelleen, my nephews - Raymond, Christopher Jr. and Michael, and my dear and
supportive friends, especially Berkele James Spar

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Abstract of Thesis Presented to the Graduate School
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THE ROLE OF THE EGGSHELL AND NEST CHAMBER
IN LOGPERCHHEAD TURTLE (*Chelonia mydas*) EGG INCUBATION

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The traditional view of parental investment in offspring has concentrated on energy expenditure by the parent in preparing propagules and nurturing the young. This study sought to expand this definition by examining aspects of nurturing that are overlooked in the traditional model, but are important in preparing reptiles because of their life history traits.

The role of the eggshell during incubation was studied as a characteristic of maternal investment. Ultrastructure of fresh and spent eggshells of the loggerhead turtle (*Chelonia mydas*) was described using scanning electron microscopy, and a pattern of band calcium-carbonate development was proposed that is consistent with known changes in developmental physiology and physical attributes of incubated eggs. Fetal calcium

investment in eggshells differed among females, and incubation in media increases of air and sand affected distribution of eggshell colours (P=0.11) without appearing to influence colours incorporation into the egg contents.

Nest nesting studies examined maternal investment in the nesting environment. Analysis of nest chamber size and structure revealed significant correlations of maternal body size and clutch size with nest size parameters. Nests dug on a reconstructed beach exhibited diverse variation in actual depth, potential air space and several shape parameters influenced by mechanics of excavation. Nests from the natural beach showed normal distributions of nest variables. The size of a chamber constructed by a female appears linked to selective pressures exerted by body and clutch size and is sensitive to variation in the density of the nesting medium. Effects of egg position within the chamber were examined by examination of nests at 3 time intervals throughout incubation and by classification of eggs as top, left, bottom or near based on location and contacts. Egg position in the nest had significant effects on egg mass, egg density, incubation time, hatching mass, and hatching sex. Significant effects of clutch on these parameters were attributed primarily to location of the nests in the beach and the accompanying thermal and hydra conditions. These findings elucidate the contribution of factors previously overlooked as important components of maternal investment and have critical implications for sea turtle nesting beach conservation measures.

CHAPTER 1 INTRODUCTION

Discussions of the concept of parental investment in offspring have traditionally centered on either transfer of energy from adult to offspring, or behavioral adaptations of the adult which culminate in extended care for the offspring. Trivers (1972) defined parental investment as, "anything done for the offspring, including brooding it, which increases the offspring's reproductive success at a cost to the remainder of the parent's reproductive success."

Denckla and Young (1982) described 2 general life-history strategies:

- 1) Species adapted to stable, predictable environments have a tendency toward greater body size, slower development, longer life span and having small numbers of young at intervals with extensive parental care given.
- 2) Species adapted to variable and unpredictable environments have high reproductive rates, rapid development, small body size and little parental care, with high mortality.

Lee further divides members of these categories clearly, drawing their babies from the second, then grows parents from the first and part of their reproductive strategy is lost positively, from the second. Taxa like fish and amphibians, with exceptions, fall into the second class, offering considerable investment in yolk stores and variable care. In marine teleost fish, 14% of known species exhibit parental care whereas 37% of freshwater

insects include care in their reproductive strategies (Krohn and Davies, 1997). The latter environment was considered to be more unpredictable, with potentially higher predation rates. Invertebrates are also generally considered members of the r-strategist and V-strategy animal group, but many species, such as insects, commonly exhibit care and brooding behavior. In invertebrate species, any contents of the propagule/brooder or embryo and any manipulations of the propagule/during or after deposition may be considered enhancements specifically for the care and protection of that embryo.

Complex and Gilbert (1990) offered a revised bipartite definition of parental investment specific to reptile eggs. The consisted of the energy invested in producing an ovum/embryo, and secondly the energy invested in means of that needed to make an embryo to be used as subsequent brooding quality.

For an animal like a sea turtle having discussions of parental investment as energy as the currency may result in serious underestimation of the actual output of resources by the female. Complex and Gilbert (1990) present the idea that the function of the eggshell in protecting the embryo might be partially analogous to the function performed by the carapace and plastron later in life, thus immediately go on to discuss parental investment in terms of yolk reserves. This represents the traditional line of reasoning that overlooks the eggshell as a type of maternal investment to ensure brooding survival. Like the yolk, the shell comes from maternal resources (protein and minerals), serves the needs of the embryo, and does not persist into later life stages.

In reviewing the evolution of the reptilian egg, Packard and Packard (1980) used theories that the eggs of the potential amniotes were similar to amphibian eggs.

with the external jelly-like layer being tough enough to serve a protective function. They suggest that calcification of the eggshell developed as a defensive measure under selection pressure exerted by microbial and invertebrate egg predators. They also describe how the amount of protective calcification is closely aligned with water absorption by the egg through incubation: eggs with minimal calcification usually require larger water uptake to supplement what is available in yolk and albumen, eggs with pliable calcified shells facultatively take up water, enhancing hatching quality, and eggs with rigid shells are generally independent of external water for developmental needs. Sea turtle eggs fall into the second class and are therefore very responsive to the incubation environment. In their discussion of maternal proteins secreted in the egg for protection and to aid in water uptake, Palmer and Goffredo (1998b) mention the variability in moisture and microbial pressures to which reptile eggs must respond. They also note the lack of parental care but do not explicitly refer to the problem as an aspect of care.

Any sort of discrimination a female turtle makes in selection of her nest site is reflected in the incubation environment of the nest. The shape and size of the chamber that she digs may also have a profound effect on the offspring by dictating the type of microclimate in which they develop. Hocking *et al.* (1983) reported significant effects of egg position within the nest on mass of hatching snapping turtles, and Savatieri *et al.* (1995) observed differential sex determination by position in grass turtle nests.

From such observations, it is clear that the egg preparation and nesting process in marine turtles have evolved to afford the eggs and offspring a type of care outside the usual considerations. The purpose of this study is to examine the alternative incubation

as maternal care made by loggerhead turtles in providing protective and adequate incubation environments for their eggs. In Chapter 2 the eggshell is examined using scanning electron microscopy, with a focus on the role it plays in incubation and the changes it undergoes in fulfilling that role. Chapter 3 quantifies the occurrence and pattern of eggshell dissolution over the course of incubation under laboratory conditions to support the observations made in Chapter 2. Nest chamber morphology regulates the natural incubation environment experienced by eggs in a clutch. Therefore, positive determinants of nest shape and size are considered in Chapter 4. Nest morphology response to nesting mode on a natural and a manipulated beach are contrasted as well. Chapter 5 considers the effect of position within the nest on a suite of egg characteristics over the course of incubation, and the effect of position on several hatching parameters. Chapter 6 provides a synthesis of the findings as a case for an expansion of the traditional concept of maternal investment in marine turtles.

CHAPTER 3
CHARACTERIZATION OF CALCIUM CARBONATE DISSOLUTION FROM
EGGSHELLS OF LOGPERCHAD TURTLES (*APSEMA CAROLINENSIS*) USING
SCANNING ELECTRON MICROSCOPY

Introduction

The shell of a reptile egg is generally composed of an inner layer of proteinaceous fibers and an outer layer of calcium-carbonate laid down over the albumen. Reptiles and birds egg usually exhibit external calcification, while eggs range from lightly to heavily calcified, and crocodilian eggs have a very thick calcareous layer (Compton and Gidley, 1990). In birds and crocodilians, these 2 materials are deposited on the egg at successively and spatially disparate portions of the female reproductive tract, whereas in turtles and tortoises, the calcare is the site where both layers are laid down (Palmer and Gidley, 1988a, 1988b; Palmer, 1990). The basic arrangement of these layers within the ultrastructure of freshly deposited reptilian eggshells has been documented by scanning electron microscopy for many species (Schleich and Kastle, 1988).

The eggshell serves a variety of functions vital to egg survival and successful hatching. Besides the protective functions cited by Compton and Gidley (1990), numerous investigations have shown that considerable amounts of the calcium consumed in the bodies of hatching reptiles are derived from the eggshell (Bischoff and Greenham,

1968, Burnard *et al.*, 1968, Packard and Packard, 1981, Packard *et al.*, 1992). The mechanism by which a sea turtle embryo draws calcium from the eggshell to meet its developmental needs is speculated to be the carbonate-sulphate-mediated formation of carbonic acid from respired carbon-dioxide and environmental water (Burnard and Grossman, 1968, M. J. Packard, *pers. comm.*). The considerable amount of calcium carbonate dissolved from the eggshell as the medium for an embryo is used for calcium, and very likely its ability for uptake (Packard, 1994). Much of this material is lost to the surrounding environment, instead the flexible nature of the calcium carbonate layer on a post-hatch sea turtle eggshell is well known by field and laboratory workers. In light of the eggshell's crucial role in protecting the egg contents from mechanical damage and microbial attack (Packard and Packard, 1980) this is a drastic alteration in structure. The visible structural changes caused by dissolution of the shell during incubation have received little attention in studies of reptilian eggs, as compared with the volume of research on eggshell formation, its structure (Packard and Packard, 1980, Solomon and Reed, 1983, Packard *et al.*, 1984, Palmer and Daddario, 1985a, Brennan and Wilbur, 1988, Packard and DeMarco, 1991, Palmer *et al.*, 1993), and mineral movement between internal egg compartments (Packard and Packard, 1980, 1981, Packard *et al.*, 1992, Packard, 1994, Packard and Clark, 1996).

The structure and composition of sea turtle eggshells have been characterized by a number of investigators. Solomon and Reed (1979, 1977) estimated morphology and biochemistry of ovalated and oviposited eggs of *Chelonia mydas*. Crystalline arrangements in formed and laid *C. mydas* eggs were described by Reed and Solomon

(1979). Eggshells of *Desmudleya carolinensis* were examined for crystal morphology (Solomon and Pratt, 1982) and resistance to fungal penetration (Solomon and Tippet, 1982). Pechard *et al.* (1982) made observations on the basic structure of *Caretta caretta* eggshells. These and other scanning electron microscopy studies have provided information on eggshell morphology. Earlier work by Jenkins (1967) and Russell *et al.* (1969) yielded data on calcium content of the shell and various egg components.

Laying down an eggshell represents a considerable investment of maternal protein and mineral resources. The significant disruption of the calcium-carbonate layer by the end of incubation suggests that egg viability considerations other than protection and calcium supply to the embryo have dictated selective processes on the evolution of the eggshell. The objective of this study was to observe pre-incubation and post-incubation shell structure of *C. caretta* eggs using scanning electron microscopy, and to evaluate the qualitative effects of embryonic use of shell resources. Specifically, this included verification and further elucidation of known eggshell structures, monitoring shells for visible evidence of the localization and sequence of calcium loss, and exploring possible causes of the late-term fragility of the calcium carbonate layer of the eggshell.

Methods

The 16 *C. caretta* eggs examined for this study were collected at Melbourne Beach, Florida, under Florida Department of Environmental Protection (FDEP) Marine Turtle Permit TP-646. Eggshells came from 1 freshly expugned eggs from different

Animals: 3 eggs from different nests hatched in situ on the beach, and 4 eggs from different females used in laboratory incubation studies. The shells from the laboratory-incubation were from 3 eggs incubated in air at 70–100% relative humidity, and 3 incubated in sand maintained at 4–7% gravimetric water content. One of the 3 eggs from each regime was incubated for 30 days, 1 for 43 days and the third until hatching.

Samples cut from all eggshells were rinsed gently in de-ionized water. Shell sections were air-dried and mounted on aluminum stubs with double-stick tape or self-healing graphite. Orientation of mounting was varied to permit other views and edge views as required. The specimens were sputter-coated with gold/palladium for 60 seconds. Shell samples were examined using a Hitachi™ S-450 scanning electron microscope. The instrument was operated at an accelerating voltage of 20kV and photographs were taken with Fujiwax™ Type 35 phosphor-screen film.

Results and Discussion

The eggshell structures observed in this study are consistent with descriptions provided by other investigators. The external mineral layer of a freshly oviposited egg (Figs. 2 + 3-3) is composed of closely packed units of calcium carbonate in the apatite aragonite configuration. The crystals of aragonite units do not interlock, but rather appear to interlock slightly at the periphery of each. This important characteristic allows the shell to be flexible, strong without damaging the calcium layer. Both rhombohedral calcium and apatite aragonite forms of calcium carbonate were reported in the eggshells of *Caprellus* (Chelmon

system by Solomon and Baur (1976) and Baur and Solomon (1977). *Corbicula* eggs and eggshells were reported to contain both crystalline morphologies as well (Solomon and Baur, 1975). The frequency and cause of both crystalline morphologies occurring in a single egg is unknown, it may be linked to diet or captivity, or a fluctuating response to abiotic environmental cues (Solomon and Baur, 1976; Solomon and Baur, 1982). In this study of *C. aspera* eggshells only the amorphous crystalline morph was observed. Deposition of the calcareous layer in the interior of the nuclei generally follows the acyral growth pattern described by Sano and Wilbur (1984) for some eggs: nucleation of calcium carbonate on a protein core to form a secondary core or locus; then growth of the initial spherulite in all directions. Internal and lateral growth of the crystal is quickly restricted by the density of the fibrous membrane and by adjacent crystal units, respectively. Therefore growth of the unit is basically unidirectional, outward from the shell.

The calcareous layer of the shell is attached to the fibrous membrane remains. From the surface of a hardened and calcium depleted eggshell, and in cross section, the membrane appear to have a sponge-like consistency (Fig. 3-3). When probed on a plane tangential to the shell surface, its fibrous nature becomes apparent (Fig. 3-4). The inner surface of the fibrous layer is lined by a thin, smooth boundary membrane of protein, visible at the lower corners of Figure 3-4. It is likely that the protein is glycosaminoglycan (Palmer and Gulleris, 1984). Solomon and Baur (1977) observed both random and parallel fiber arrangements in the interiors of *Chironomus* species. The same was found in *C. aspera* eggshells in this study. From a mechanical standpoint, this arrangement appears to

extensive bubbling and changes in rotational velocity of the egg as water in the fibres are lost down, similar to that described by Palmer *et al.* (1991) within the context of *Leptogomus* eggs. The individual fibres consist of a polysaccharide sheath with a central protein core (Solomon and Reed, 1977). No differences were observed in the fibrous membrane layer of fresh, midtime and hatched eggs. It is possible that some change may have been overlooked as an artifact of the preparation process (air-drying) because Pankard *et al.* (1984) observed no differences in morphology between air-dried and critical-point dried eggshells of *Encrinurus glaucus*. From personal observation, there is an increased pliability of the fibrous layer when it is hydrated; dry eggshells are extremely brittle. Rehydration of dried eggshells restores much of its pliability and tensile strength. Whether any swelling of the fibres, or the membrane as a whole, occurs in the presence of water is unknown. An alternative preparation technique such as critical fixation of fresh eggshells in glutaraldehyde prior to drying may elucidate this. Any changes in membrane pliability and thickness due to hydration would have important consequences for function of the calcareous reticulate layer, particularly in eggshells where some depletion has occurred.

The points of attachment between the calcareous layer and the fibrous layer are the mammillae, protein cores that serve as nucleation sites for crystals as the eggshell is being formed (Figs. 3-3, 3-4). Solomon and Reed (1983) observed the same mammillary layer structure for hatched *reticulolamprolaima* (*Reticulolaima nemataleptum*) eggshells as that seen for logarithmic eggs in Figure 3-4. The configuration of aragonite crystals radiating from the mammillae reflect the pattern of calcium carbonate deposition onto the

membrane core crystal. The spent eggshells from the sand and mineral isolations/baths showed a preponderance of empty membrane (estimated > 90%) with scattered crystals from lost crystalline units during and penetrating the testates (Figs. 2-5, 2-7). The calcium carbonate layer in the air-isolated egg was much less friable. It could not be ascertained whether basal structure observed by manual flaking of the mineral layer from the air-isolated-eggshell was authentic or a preparation artifact (e.g. empty membrane and degraded crystalline bases). When the calcium carbonate layer of the fresh eggshell was fractured from the testates, the bases of individual crystalline units, bearing the characteristic shape of the membrane, could be observed (Fig. 2-8). A row of the units runs diagonally across the figure, and their increasing size from the proximal to the outer, distal end can be seen. Additionally, a membrane core with some associated crystal growth can be seen protruding from the surface of the testates at the left, just below center.

No profound structural differences were observed between shells of eggs incubated in sand and air for 30 and 45 days, and fresh eggshells (except for a slightly more weathered appearance in the 45 day sand-isolated eggs). Penetrating the shell at the mineral layer to membrane interface (as done for Figures 2-8) resulted in preparations that were not easily distinguishable from those of fresh eggshells. At hatching, mineral sand and air-isolated eggshells exhibited ready flaking of calcium carbonate patches from the shell surface under minimal mechanical duress. This condition required attempts to photograph the mineral-free core layer interface and mineral layer surface at hatching, due to moisture charging and specimen damage under the electron beam.

Brinkman and Mollath (1988) described mammillary cores in vertebrate eggs and the manner in which crystalline shell units grow upon them, as discussed earlier. The cores themselves and the apical crystal growth upon them can be seen clearly in the naturally-moulded spent eggshell pictured in Figures 2-9 and 2-10. Most of the calcareous carbonate layer had already leached off of the fibrous layer in these preparations. The most cores within the mammillae and associated crystal structure seen here were not commonly observed in shells of hatched eggs, but were found scattered sparsely on 6 of the 7 in size eggshells and the shell of the egg incubated to hatching in air. Usually, the mammillae were hollow as in Fig. 2-4, indicating that the core crystals were either completely dissolved or partially dissolved and leached then lost. The 2 core-crystals in Figure 2-10 (1 in the center of the mammillae and the other behind and to the left of it) exhibit little remaining contact with their surrounding apical crystals, suggesting that some amount of dissolution has occurred.

Mammillary cores clearly provide a foundation for crystalline unit development and anchor the individual units to the matrix (Solomon and Reed, 1983; Pridmore and DeMarco, 1991). Upon examining the hatched eggshell structure and the differences seen between fresh and hatched eggs in light of the proposed mechanism for calcareous carbonate dissolution, a number of observations emerge. The extreme fragility of the eggshell's mineral layer is not evident until late in incubation and as noted earlier, profound visible changes are not visible until after 45 days. It therefore follows that the crystalline units are mainly intact up to this time, with slightly spaced/proximal ends and inter-based distal ends. This configuration would support both the pooling of surface fluids from neighboring

expansion in the dead air spaces close to the fibrous layer, and retention of water by capillary action in the same spaces. With carbonic anhydrase available from the chondroitinase membrane, this lower area of the calcareous calcitonin layer are likely sites for dissolution to occur.

If the primary cause of dissolution is the water base of the individual calcitonin, the scaling that occurred would weaken connections to the overlying structure. Alternatively, the core crystalline plugs act within the 'sockets' of the membrane (Figs. 2-4, 3-10), affording intimate contact with the membrane residues and eventually accounts for the demineralization. Some partial dissolution of the core, as seen in Figure 2-15, destroys its connection with the apical crystal growth. Either of these scenarios would result in a profound loss of structural integrity in the 'foundation' of the calcareous calcitonin layer while releasing expansion and warping at the dead ends of the crystal units. An eggshell in this condition would readily display the observed characteristics of poorly coupling of calcareous material from eggshells when very close to handling and in shells from hatched eggs. Such a pattern of dissolution allows the continuity of the dead mineral layer to maintain protection from dangers such as fungal and microbial invasion. Additionally, dissolution of the core observed late in incubation may result in weakening the integrity of the membrane residues, thereby facilitating popping by the hatching.

The suggested pattern of calcareous calcitonin dissolution from sea turtle eggshells is consistent with the theoretical mechanism of mineral solubilization, the observed shell structure and changes occurring during incubation, and post-incubation observations of shell fragility. The mineral and fibrous layers of the eggshell are clearly a mineral

movement in case of the embryo-fertilization mechanism, the former layer must act as it provides protection, material for growth, and appears to be responsively altered by the embryo as a response to its needs.

The scanning electron microscopy technique used for this study provides useful qualitative information about initial environment and subsequent changes in the eggshell (Chapter 3) measures dissolution of eggshell-calcium-carbonate quantitatively, with attention to the magnitude of the initial environment, and the timing and localization of eggshell resource depletion in different incubation media.

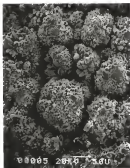


Figure 3-1 External surface of Bath Rogeria (*Cervini corvici*) eggshell. Each nodule of calcareous material is the distal end of a structural unit of the calcareous carbonaceous layer.
X 750

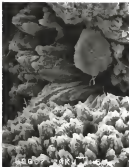


Figure 3.2 6X magnification of central portion of Figure 1. Note various orientations of enstatite crystal polymorphs of calcareous surfaces and contact between crystals of separate nodules. X: 4000



Figure 3.3 Filter membrane (reticulate) of hatched egg. Edge of sample with 'spongy' appearance and loss of calcium-carbonate layer from bottom. X 750

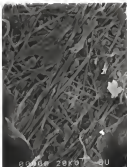


Figure 3-4 Amorphous filter layer of wood-based lattices (internal to boundary layer, seen at lower left and right) showing irregular arrangement of protein filers. $\times 4000$

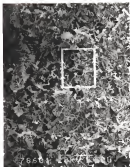


Figure 3-5 Internal surface of hatched egg. Most of the calyx layer has sloughed off leaving bead structure and scattered crystals. Microfolds with radiating crystals is visible under lower edge of highlight box. X 1500

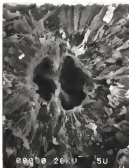


Figure 2-4 Internal surface of hatched egg. Detail of ommatidia (darkened area) on shell membrane. Note radiating pattern of crystals, depth and shape of 'socket' for each crystal. X4000

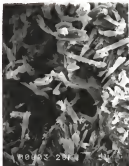


Figure 3-7 Magnification of Figure 3-6 (500) showing remnants of the crystalline layer and underlying fibers of amorphous nanoscale. X 1750

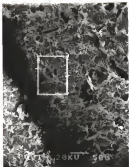


Figure 2-4 Internal view, colorless layer of fresh eggshell with membrane tentacles peeled away. Note that many rows of nucleation are in order to maximize resources (cf. Figure 2-6). Tapered bases of crystal units can be seen with spines around them. Inset cone in the tentacles is a hole just below center. X 750.

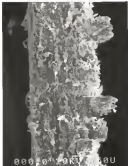


Figure 2-6 Edge of etched basketed eggshell showing 3 secondary coats at different rates of degradation. Note mineral calcium carbonate remaining on shell between coats X 750



Figure 2-13 Edge of naturally-modified hatched eggshell (pivot of Figure 2-8). Cross section of intact secondary core. Note similarity of radiating aragonite crystals at left center to the structure in Fig. 2-6: space between core and apical crystals, and smoothed appearance of core crystals. X 1150

CHAPTER 1 EGGSHELL CALCIUM DISSOLUTION IN ARTIFICIALLY INCUBATED LOGGERHEAD TURTLE (*Caretta caretta*) EGGS

Introduction

Chapter 2 discussed the importance of the sea turtle eggshell as a mineral incubation, its structure, and the mechanism by which it is dissolved to facilitate development and hatching. While eggshell is to be considered an investment in the care of the embryo, it is important to know the magnitude of this investment.

Early investigations of calcium metabolism in marine turtle eggs demonstrated that the eggshell acts as a substantial contributor to embryonic development. Benson (1962) observed that 75% of the calcium in leatherback hatchlings (*Caretta caretta*) came from outside the egg contents. He etched the eggshell and measured its possible mineral reserves. Bosted and Greenbaum (1964) tested the latter hypothesis and found that alkaline levels at green turtle (*Chelonia mydas*) nests were so low as to preclude absorption of calcium and other minerals from sea water present in the medium or taken in by the egg. They advanced the idea that dissolution of the eggshell or surrounding calcium carbonate sand by carbonic acid could provide calcium to the embryo. Bosted et al. (1964) incubated loggerhead (*Caretta caretta*) and green turtle eggs in calcareous coral sand and relatively inert silica sand. No significant difference in calcium content of

the hatchlings was found between the 3 treatments, thereby implicating the eggshell as the major calcium source. The relative contribution of the eggshell to the embryo was found to be 62%. Recent studies of calcium mobilization in reptile eggs place the chorionic eggshell contribution to hatching calcium at about 70% (Packard, 1984; Packard and Clark, 1985).

The pattern of increased calcium dissolution late in incubation observed in Chapter 2 has been described for numerous reptilian species from the viewpoint of embryonic use of shell resources (Packard, 1984) but little attention has been given to the calcium carbonate that remains on the eggshell at hatching. This is a substantial amount in turtle eggs and represents the maternal resource investment in protecting each embryo. Given the role of the hyaline and gelatinous membranes in directing the eggshell, it is clear that carbonate remains would be a principle determinant of quantity of residual calcium carbonate. The aforementioned laboratory incubation performed by Huxard *et al.* (1969) gave no indication of whether eggs were buried or grouped individually, or only partially buried in the media used; it is possible that differential dissolution according to conditions may result in differential incorporation of calcium by the embryo. The decalcification mechanism of the embryo accomplished both mineral and water uptake and gas exchange (Thompson, 1983; Packard, 1984). It was hypothesized that as areas of advanced membrane development and vascularization, such as the upper region of the eggshell during the middle third of incubation, greater loss of calcium carbonate might occur.

The study was designed to elucidate the role of the eggshell calcium carbonate layer as well as examine the changes it undergoes during incubation. Aspects explored

were the amount of the initial movement in the calcium carbonate layer of the eggshell, the amount and timing of dissolution of calcium from the layer, effects of the incubation medium on mineral loss, and the location of mineral loss on the eggshell surface. Litter mass of hatchlings, and calcium content of hatchlings and yolk were examined as possible indicators of differential developmental quality conferred by incubation at varying media.

Methods

Loggerhead turtle (*Caretta caretta*) eggs for use in this study were collected from Melbourne Beach, Florida, under permit number TP-534 from Florida Department of Environmental Protection. Five nesting females were located on one night, and 30 eggs were collected from each. In each case, for 30s through the 30th eggs were taken as maximum possible variance in calcium content among eggs dropped early or late in laying. All sample eggs were caught as they fell from the cloaca and placed into egg cartons for transport back to the incubation area. Maximum diameters for all 100 eggs were recorded and total surface area was calculated for each. Figure 1-1 outlines the experimental design. Two eggs from each of the 5 clutches were terminated within 12 hours of collection for analysis of fresh egg samples. The remaining 90 eggs were incubated at 28.5° C. Nine eggs from each clutch (45 total) were incubated *in air* on plastic media suspended above water filled trays. The trays were covered with plastic and were maintained at 90-100 % relative humidity. The other 5 eggs from each clutch were incubated in plastic-covered, sand filled trays, with 1-3 cm of sand covering each egg.

Osmotic water content of the sand was maintained at 4-5% throughout the incubation period. Air stones attached to standard aquarium pumps and inserted through slots in the plastic covering over the trays helped to maintain air circulation and humidity in both incubation regimes. The air stones were immersed in the water or laid on the sand surface.

At 30 and 40 days of incubation, 10 eggs from each medium were removed to measure eggshell calcium content. Sand was removed from the sand-incubated eggs by gently rinsing in deionized water before processing. The remaining 23 eggs in each medium were allowed to hatch. All eggshells were analyzed for calcium content.

Hatchlings were weighed and released as hellgramites fresh within 3 days of hatching. The same overall experimental protocol was applied to a second collection of 100 eggs with one modification. 28 hatchlings from each incubation medium were killed at time of hatching, and analyzed for yolk and hatching calcium content. Of those marked for incubation, 19 eggs from the air incubation regime and 17 eggs from the sand incubation hatched. Upon emergence from the egg (yapping), hatchlings were killed by hypothermia, the yolk sac and any internalized yolk was separated from the hatching. Only the fresh yolk (19 eggs) and the residual internalized yolk and hatching calcium content data from the second incubation were included in the current study.

To determine eggshell calcium content, 4 samples (each 3 mm in diameter) were taken from each shell. The 4 samples were taken from a lineal across the fresh eggs. Three samples were taken from the upper hemisphere of shells (antral pole of the yolk), and determined by incubation (antral) from incubated eggs, and 1 from the lower

hemispheres. Each sample was dried to constant mass (<10 h) at 100°C . Dry mass was recorded and samples were ashed for 12 h at 450°C in a muffle furnace. Ash mass was measured.

Each ash sample was digested in a maximum of 12 M HCl and diluted up to an appropriate volume with de-ionized water. A subsample of each was brought to a working dilution between 0 and 0.125 meq of calcium with de-ionized water and inductium chloride, to be read as a linear standard curve calibrated at 0, 0.052, and 0.125 meq. Calcium content of the final dilution was measured by flame analysis, with averaging of triplicate readings using a Perkin Elmer 2150 Atomic Absorption Spectrophotometer. Total calcium content of the original samples was calculated based on the dilution factors of the original samples. Total calcium per eggshell was calculated by dividing a multiplier from dividing shell surface area by sample surface area. To investigate the possibility of differential dissolution from different areas of the eggshell, masses of the 3 samples from the lower and upper hemispheres of the egg were taken and used to calculate calcium content for each half of the eggshell. Yolk and hatchling calcium determination was done by drying the samples to constant weight at 100°C (<10 hrs), ashing in a muffle furnace at 450°C (<10 hrs), then following the same digestion and dilution method used for the eggshell samples.

Differences in calcium concentration in eggshells among females were analyzed by ANCOVA (SAS PROC GLM) (SAS Institute Inc. 1988). The effects of clutch, incubation method, and length of incubation on eggshell calcium content were also examined using ANCOVA. Upper and lower eggshell hemisphere calcium contents were compared both

warm media and across media, throughout the incubation period. To control for differences in total eggshell calcium due to egg size, all analyses were repeated using mg of calcium per cm^2 of eggshell (total eggshell calcium/eggshell surface area). Last mean of the hatchlings was contrasted between the media for possible differences due to incubation regime and calcium availability. Yolk and hatchling calcium content were tested for effects of clutch and incubation medium (ANOVA).

Results

The calcium content of the shells of freshly-laid eggs was significantly different among individual clutches ($P < 0.01$) (Fig. 3-2). Clutch means ranged from 176 to 312 mg of calcium per eggshell. The sample size for the evaluation of initial calcium content was only 3 eggs per clutch. The persistent pattern of clutch mean responses for eggshell calcium content through the experimental groups and over time, however, strongly supports the results of the fresh egg analyses (Figs. 3-3, 3-4).

Eggshell calcium content did not vary significantly between the air and sand treatments for the first 45 days of incubation (Fig. 3-2). After day 45, there was a rapid increase in dissolution of calcium from the shells of the sand-incubated eggs, resulting in a significant difference in calcium levels at time of hatching ($P < 0.05$). Mean total calcium lost was 17.2 mg for air-incubated eggs, whereas sand-incubated eggs lost 40.2 mg (mean for fresh eggs, mean total eggshell calcium content at hatching for air and sand, respectively). There were significant responses of calcium content to clutch, incubation medium, and length of incubation, and secondary interactions among all 3 variables in the

usual ANOVA. Subsequent ANOVAs sorted by age and medium sorted examinations of the sample effects by clutch. Clutch C exhibited no significant change in eggshell calcium content over the course of incubation in air (Fig. 3-3). Initial and final eggshell calcium content differed significantly within the other 4 clutches ($P < 0.05$). Incubation in sand yielded significant changes between initial and final calcium content in all clutches except Clutch D (Fig. 3-4).

No significant differences in calcium dissolution from different areas of the eggshell were observed ($P > 0.05$) (Fig. 3-5). In both media, air and sand, upper and lower hemispheres of the eggs lost calcium at similar rates. The secondary analysis using mg of calcium per cm² of eggshell yielded results that differed in magnitude but not in significance levels or interactions from the analysis using total eggshell calcium. The latter are presented in the results and discussion for ease of comparison with values from the literature and for greater biological significance in most cases. The level of hatchlings was significantly different between 2 of the clutches ($P < 0.01$) (Table 3-1). However, there was no significant effect of incubation medium on live mass.

Fresh yolk calcium content did not vary significantly among the 5 clutches ($P > 0.05$) (Table 3-2). Incubated yolk calcium content was significantly lower in 2 of the clutches ($P < 0.05$). There was no effect of incubation medium on calcium content of zoned yolk. Hatching calcium content in only 1 of the 5 clutches - clutch F - exhibited a significant response to incubation medium. Mean calcium content of hatchlings from air-incubated eggs of clutch F (133.29 ± 1.74 mg) was less than calcium content of hatchlings from sand-incubated eggs (139.87 ± 1.83 mg) ($P < 0.05$).

Discussion

Initial Calcium Content and Amount of Calcium Deposition

The amount of calcium stored in each eggshell of a freshly laid leghemoglobin egg appears to vary consistently among individual females (Fig. 1a,b). The values for fresh eggshell calcium content found in this study were lower than the value published by Howard *et al.* (1967) (31–222 mg compared to 242 mg). The value from the earlier work is based on examination of 5 leghemoglobin eggs, from a different population (Illinois Island, Australia), with a slightly different method of initial sample preparation (nitric acid peroxide acid digestion, rather than hydrochloric acid). No indication was given as to whether the 5 eggs were from the same clutch or different females.

There are no previous data available on eggshell calcium variation within a clutch or within an individual female's successive clutches over the course of a season. The variation in eggshell calcium observed among individual females is probably not due to the number of previous nestings that season, as several pools for egg-shedding do not seem to be a limiting factor (Skelton, 1962). Factors that may influence initial calcium deposition on the eggshell are the shedding efficiency of the individual female, egg retention time in the uterus, and clutch size. Auker and Selman (1976) described the shedding of the green turtle (*Chelonia mydas*) and postulated deposition of calcium at the same site as mammary secretions. Work on *Cryptorhina polyphaga* (Palmer and Guellette, 1984) confirmed this sequential deposition rather than reproductive tracks. Length of egg

retention in the region would clearly affect eggshell calcium content in fresh eggs. The variability in thickness of the eggshell's calcium layer must fall well within the margins of being too thin to supply and protect the embryo, and being too thick to allow sufficient depletion for pipping at hatch time.

The fragile nature of spent eggshells required that care be used in handling of the eggs for the analyses. It is possible that some of the calcium loss observed was due to the abrasion nature of the sand incubation and loss in rinsing and handling of eggs from both media. However, no visible particles from the material layer remained in the sand, and there was no evidence of calcium-carbonate particles in the deionized water used for rinsing the eggs. When the material layer of air-incubated eggs was intentionally abraded in run for durability, it exhibited far greater structural integrity than the material layer of sand incubated eggs. The level of acidity is consistent with a lower amount of dissolution having occurred in the lowermost level of the material layer as discussed in Chapter 3. It is therefore likely that the observed levels of calcium loss are legitimate and loss of shell material in preparation of the samples was negligible. The difference between air and sand incubation in loss of calcium was significant. The fact that almost 3 times as much calcium is lost during sand incubation probably reflects 2 characteristics of the sand incubation micro-climate. The lower availability of usable water (Pickard *et al.*, 1981) and the greater air circulation imposed by lack of other moisture sources to limit carbonic acid formation and subsequent dissolution of calcium from the shell.

Pattern and Location of Mineral Loss from the Eggshell

Differences observed in dissolution patterns among clutches (Fig. 3-3, 3-4) are possibly due to differences in the density of the calcium carbonate matrix. As noted in Chapter 2, eggs from different clutches appear to have varying densities in terms of spacing of the calcium carbonate aragonite matrix. These features probably limit gas dissolution and water infiltration in eggshells of some clutches. The difference in structure could also explain the lack of significant calcium dissolution in eggs incubated until hatching for some clutches. Packard (1974) noted that differences in shell structure among reptile species did not cause substantive differences in egg content calcium in eggs of similar ages. Therefore, the relatively small interspecific differences in structure would not be expected to cause variation in internal egg calcium compartments. The amount of eggshell calcium remaining after incubation could, however, be expected to vary with structure, based on the mechanics of dissolution described in Chapter 2.

The data obtained in this study support the observations made by scanning electron microscopy in Chapter 2: the bulk of calcium carbonate dissolution from the eggshell occurs toward the end of incubation (Fig. 3-3, 3-4). This pattern is consistent with the higher levels of calcium malonate observed late in incubation for *Leptodeira* and *Sceloporus* (Packard and Clark, 1976). Most snakes and lizards utilize their considerable yolk stores of calcium for early embryonic growth and depend on eggshell calcium in the final week-end of incubation; alligators and birds generally use yolk calcium

during the first half of incubation and tap into eggshell calcium during the second half' (Packard, 1994)

As noted in the previous chapter's discussion of eggshell calcium depletion, carbon dioxide from embryonic metabolism combines with water in the environment to form the carbonic acid which solubilizes the eggshell calcium (Steward and Greenham, 1994). Ackerman and Prange (1971) documented the increase in carbon dioxide levels and decreasing oxygen tensions that begins several weeks before hatching. It is the increase in carbon dioxide output and the greater availability of water from substrate contact (Hyndes et al., 1983) that likely accounts for the increase in calcium dissolution in semi-incubated eggs after 45 days of incubation (Fig. 3-3). Whereas alligators and some species of fresh water turtles show a peak followed by a decline in embryonic oxygen consumption very late in incubation, marine turtle embryos exhibit little decline in the rate of oxygen consumption (Thompson, 1989). Thompson explains the protracted consumption pattern as a mechanism for facilitating synchronous hatching. Sustained high respiratory rates by the sea turtle embryos also increases carbon dioxide levels in the nest. This may provide a final carbonic acid "pulse" to render the eggshells more amenable to pipping.

Calcium carbonate dissolution appears to occur homogeneously over the egg surface. Neither air nor sand incubated eggs exhibited location-specific differential loss of calcium carbonate from the shell (Fig. 3-4). This indicates that the micro-environments imposed by these media mixtures are uniform and result in equal dissolution from all areas of the shell. The micro-environments occupied by eggs in natural nests are far more variable in water and gas relations (Coughlin and Calhoun, 1990). An egg incubated in a

heterogeneous micro-environment such as the periphery of the nest might exhibit localized areas of high desiccation, based on the nest and air effects observed in this study. The nature of the calcareous carbonate layer of the eggshell with its specific arrangement of aragonite crystals, however, could possibly allow wicking of a larger area of eggshell by capillary action from a point contact with wet substrate. Further experimentation with controlled contact points could determine whether this capillary wicking occurs.

Marshall (1990) noted high levels of clutch mortality associated with very dry nesting conditions on the green turtle (*Chelonia mydas*) rookery at Aqueduct Island. A portion of this was late stage mortality – hatchlings peeping but failing to emerge (Marshall, pers. comm.). If the dry conditions are precluding or limiting formation of substrate and water the nest chamber, the calcareous carbonate shell layer may not be sufficiently depleted, and actually confine the hatchlings inside the eggs. If the calcareous morphology of calcareous carbonate occurs in eggs of natural green turtle populations as it did in the farm-raised eggs of Solomon and Board (1976), the problem would be compounded since the tubular calcareous crystals form a stronger mineral matrix.

Hatching Sites, and Yolk and Hatching Cholesterol Content

Incubation environments which allow substrate contact can increase the availability of liquid water to eggs by contributing the capillary water held around the particles of media (Puckard and Puckard, 1980; Puckard et al., 1981). Gotsch et al. (1987) and Thompson (1987) demonstrated fresh water turtle hatchlings from eggs incubated on wetter substrates as being larger based on greater utilization of yolk resources. In the current study, live hatching mass did not vary significantly between the incubation

moisture (P=0.05) (Table 3-1), despite the apparent availability of more usable water to the sand incubated eggs. Resident yolk from 2 of the clutches did contain less calcium than yolk from the others, however, this appeared to be a significant effect of clutch rather than incubation medium (P=0.00). When added to the observation that only 1 clutch exhibited incubation medium-related differences in hatching calcium content, it suggests that offspring from different clutches may possess distinctive efficiencies in calcium mobilization. Packard (1984) reported that the availability of water to the embryo in diadromic eggs did not seem to affect internal calcium balance. This indicates that calcium is not limiting in embryonic development and is probably not excluded in the moisture-mediated utilization of yolk resources discussed by Crotts *et al.* (1987) and Thompson (1987). Normal to somewhat incubation apparently make sufficient eggshell and yolk calcium available to the embryo. The degree of exogenous calcium loss by the eggshell is regulated to a greater degree by the hydroic conditions of incubation.

This study examined eggs that were incubated in conditions not unlike the 3 extremes that might be found in a natural nest: an egg at the upper or lower edge of a nest chamber, almost completely surrounded by sand; and an egg in the center of a clutch, in contact only with other eggs and air. Within a natural nest, however, eggs are exposed to a range of contact situations which can denote a continuum of microclimates. To better understand the micro-environments that exist within a clutch incubating on the beach, information regarding nest chamber size and shape was necessary. In Chapter 4, natural sea turtle nests are characterized morphologically in order to investigate how the incubation environment is prepared by the female in optimal clutch success.

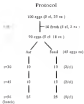


Figure 3-1 Distribution and termination schedule for 100 logarithmic-(C. cornuta) eggs moulted on air and sand

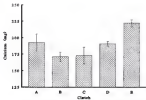


Figure 3-1: Calcium content (µg/g) of eggshells from five different clutches of the Loggerhead turtle (*C. caretta*). Values shown are mean \pm SD.

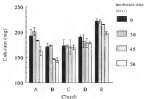


Figure 2.3 Eggshell calcium content for 5 clutches of Loggerhead eggs incubated in air. Bars for each clutch represent the calcium content of eggs incubated at the respective incubation times. Values shown are means \pm SE.

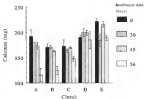


Figure 3-4 Egg shell calcium content for 5 clutches of loggerhead eggs incubated in sand. Bars for each clutch represent the calcium content of eggs terminated at the respective incubation times. Values shown are means \pm SE.

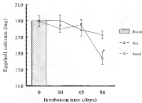


Figure 3-5. Eggshell calcium loss (mg) of loggerhead turtle eggs incubated in air and sand. Values shown are means \pm SE. Calcium content of sand incubated eggshells is significantly lower at hatching ($P < 0.05$) as indicated by *.

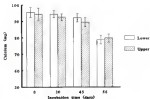


Figure 3-4. Eggshell calcium content change (mg) by position (upper or lower hemisphere) of the egg through the incubation period for feggshead variety eggs. Values shown are means \pm SE. Calcium content at 84 days is significantly less for both upper and lower hemispheres ($P < 0.05$). Upper and lower hemispheres do not differ significantly from each other.

Table 3-1. Mean hatchling mass (g) by clutch and incubation medium. Data are given as mean \pm SE. Hatchlings from Clutch B and Clutch D are significantly different ($P < 0.05$). Differences between air and sand incubated hatchlings were not significant.

Clutch	n	Mean body mass (g)
Clutch A	8	09.50 ± 0.42
Clutch B	8	$13.80 \pm 0.38^{**}$
Clutch C	9	09.47 ± 0.42
Clutch D	6	$20.11 \pm 0.42^{**}$
Clutch E	7	20.00 ± 0.45
Air hatchlings	31	09.70 ± 0.26
Sand hatchlings	20	09.10 ± 0.27

Table 3-2 Mean calcium content (mg) of fresh yolk, residual yolk at hatching, and hatchlings from air and coal-mineral-lagoated (C) cowbirds eggs. Inter-clutch differences in fresh yolk and hatching calcium content are not significant ($P > 0.05$). Residual yolk calcium content is significantly lower in clutches 1 and 3 (*) than in the other 3 clutches.

Clutch	Fresh yolk	Residual yolk	Hatchling
1	111.12 \pm 0.80 ($n=5$)	19.19 \pm 0.23 ($n=5$)	131.48 \pm 3.43 ($n=5$)
2	104.51 \pm 0.86 ($n=5$)	18.90 \pm 0.21 ($n=5$)	146.39 \pm 0.66 ($n=5$)
3	109.91 \pm 0.36 ($n=5$)	18.69 \pm 0.38 ($n=5$)	135.70 \pm 1.96 ($n=5$)
4	106.94 \pm 0.38 ($n=5$)	15.15 \pm 0.38 * ($n=5$)	135.83 \pm 1.57 ($n=5$)
5	107.11 \pm 1.26 ($n=5$)	15.17 \pm 0.27 * ($n=5$)	139.07 \pm 3.25 ($n=5$)

CHAPTER 4 NEST CHAMBER MORPHOLOGY: RELATION TO FEMALE BODY SIZE, CLUTCH SIZE AND NESTING MEDIUM

Introduction

The environment experienced by a clutch of sea turtle eggs during incubation is determined by the shape and size of the nest chamber a female excavates. The physical attributes (temperature, humidity and gaseous regimen) of the nesting beach interact with the clutch based on the types of contact points, gas gradients, and thermal and hydric connections available within the chamber. The results obtained in Chapter 3 underscored the role that contact with the incubation medium can play during egg incubation. Many effects of physical factors on individual eggs and clutches have been documented for burial oxide eggs (Miller 1940; Mironovskiy, 1942; Wysocki *et al.*, 1984; Ackerman, 1991; Packard, 1992). Much of the investigation of the incubation chamber of sea turtles has been limited to studies of nest-digging behavior (Watanabe and Kawato 1992). As noted in the review by Cruz *et al.* (1993) the manner in which the beach is perceived by a nesting female, and the subsequent effect that the type of chamber she prepares has on her clutch can be greatly influenced by beach circumstance. Mortimer (1996) documented

the effects of sand quality, especially grain size, on the digging success of nesting green turtles (*Chelonia mydas*).

This study was designed to explore female body and clutch size parameters and their possible correlations with nest size parameters and to measure the effect of the nesting medium on chamber size and shape by comparing a natural beach to a moderated beach. The lack of definitive information concerning the architecture of natural nest chambers prevents the informed accumulation of data from incubation characteristics seen in laboratory study. A knowledge of the size and shape of a natural chamber would help for instance, in estimating how many eggs might be held in different positions. Whether nest cavities are uniform in their proportions and volume of air space available to a clutch of a vital link in understanding the environmental factors affecting incubation. Campbell and Gilmore (1996) noted the importance of understanding the degree to which a female can control or manipulate the nest micro-environment in furthering our knowledge of reproductive ecology.

Materials and Methods

Nest chamber morphology was examined by use of excavation and casting techniques developed in the course of the study. A total of 51 Loggerhead (*Caretta caretta*) nests was utilized in the investigation. Thirty-five of the nests were from Melbourne Beach, Florida, and the other 16 were from the beach at Patrick Air Force Base, approximately 13.8 km north of Melbourne Beach. The Patrick Air Force Base

beach was the site of an extensive sand recruitment project completed 3-5 months prior to the onset of the nesting season, whereas Midbourne Beach was representative of a natural nesting/beach condition, having undergone natural erosion and deposition of sand prior to commencement of the 1991 nesting season. There are no records of the areas ever having been researched. These locations will hereafter be referred to as natural (Midbourne) and researched (Patrick Air Force Base).

Loggerhead incubation stages of nesting activity ranging from entering the water to last nest chamber excavations were located. All were permitted to proceed without interference until 20-40+ eggs were deposited. Sand around the posterior 1/3 of the animal's body was removed down to the level of the body pit, with the posterior edge of the plastic as the body pit depth standard. The characteristic flexion of the rear flippers that accompanies deposition of each egg was used to help place a 122 x 61 cm sheet of 1.3 mm thick plastic under the flippers and rear body. A U-shaped cutout in one long edge of the sheet was positioned under the cloaca to allow excretion to continue unimpeded. The plastic was brushed free of sand and prevented the turtle from sweeping sand into the nest chamber. Orientation of the animal (facing down-head, angled, etc.) was noted and marked in the field notebook, used for later reference. At the first covering sweep of the last flippers, the animal was lifted by 2-3 people and moved forward 1-2 m. The nest cavity was covered by a 30 cm square of plastic sheathing. Once clear of the nest, the animal was lifted again, moved laterally and turned to face seaward to prevent her from obstructing the nest. Straight carapace length (SCL - nuchal notch to pygal notch) and straight carapace width (SCW - widest point) were measured to the nearest 0.1 cm with

large aluminum calipers and the animal was checked for tags and injury. Length measurements of both rear flippers were taken from the proximal end of the rib to the distal tip of the longest phalange. The mean of these 2 rear flipper measurements was taken as the mean rear flipper length (MFL) for each animal.

At the nest site the small covering sheet was removed and a plastic liner (composed of 2 m of string tied at each end at the centers of 0.5 m-diam holes) was placed across the cavity opening. The line was placed perpendicular to the head-tail axis of the nesting turtle, with the string tied to each end resting on the sand surface just outside the body pit. This gave a beach surface reference point for nest depth measurements. Body pit depth (= beach surface to posterior phalanx resting point) and minimum egg depth (= beach surface to top egg or nest) measurements were made.

A layer of thin plastic food wrap was laid on top of the eggs in the chamber conforming to their topography. This was followed by dampened paper towel or tissue to allow the casting material to cure. Great Stuff® (a polyurethane expanding foam product) was applied to the prepared chamber to make a cast of the cavity nest. The chamber was covered with newspaper to exclude sand and debris. After curing for 3 hours, the neck nest was marked with the projection of the animal and footprints were inserted around it to mark the sand surface (at body pit depth) (Fig. 4-b,c). The cast was then removed with care not to disturb the cavity nest, and the footprint line was drawn in with indelible marker. All eggs were removed from the nest chamber and counted. Ten eggs were chosen at random and measured for maximum and minimum diameters. Egg volume was calculated as an ellipsoid ($V = \pi/6 ab^2$), where a = maximum diameter, b = minimum

diameter). The mean volume for the 18 eggs was multiplied by the total number of eggs in the clutch to obtain clutch volume.

Nest cavity depth was measured from the plastic line to the bottom of the cavity with the back end of the large calipers. Polyurethane foam was applied to make a cast of the entire nest cavity, allowed to cure, marked for nest surface and orientation, then excavated (Fig. 4-1b). Actual nest depth was calculated by subtracting body pit depth from nest cavity depth. Volumes of the neck cast and cavity cast were determined by measuring water displacement on immersion of the casts in the trough surface line. The mean of 3 displacement volume measurements was taken for each cast. For the rest of the methods, results and discussion, 'neck' will refer to the upper, constructed part of the cast or cavity ('chamber' will refer to the lower, natural part of the cast or cavity). On the full cast, chamber width and length, and neck width and length were measured. The neck and chamber measurements were taken from the narrowest and widest points on the cast, respectively (Fig. 4-2). For each cast, clutch volume and neck volume were subtracted from the full cavity volume to obtain potential airspace within the nest. Cross sectional areas were calculated for the chamber and neck of each cast using the width and length measurements. The neck area was subtracted from the chamber area to measure how much the parts of the cavity overlapped.

All linear measurements were recorded in cm and volume measurements were taken in ml. Correlation analyses were done between nest chamber measurements (body pit depth, nest cavity depth, actual nest depth, minimum egg depth, chamber length, chamber width, neck length, neck width, neck volume, cavity volume, and airspace), and

the 2 other groups of measurements: female size (SCL, SCW and MPL) and clutch size (number of eggs, mean egg volume and clutch volume). Correlation was performed as a modelled regression using SAS PROC GLM (Littell et al., 1991) to obtain initial R^2 values, with and without location specific effects.

Nest chamber size parameters were compared between locations by ANOVA (PROC GLM for an unbalanced design, using SCL as a covariate) and subsequent Levene's F-test for Homogeneity of Variances (modified PROC TTEST) (Conover et al., 1981; Littell et al., 1991).

Results

The female size parameters (SCL, SCW and MPL) and the clutch size parameters (number of eggs, mean egg volume and clutch volume) were significantly correlated with several parameters of nest depth or size (Table 4-1) (* indicates significant correlations $P < 0.05$). In Table 4-1, for each correlation, the upper value indicates the proportion of observed variability that location helps to explain, and the lower gives the proportion of variability explained by non-location specific effects. Relatively low R^2 values for all correlations were further reduced or were equal to zero (omission of location specific effects). The strongest correlations (some of which exceeded $R^2 = 0.31$) were found between measures egg depth and number of eggs, air space and SCW, and width of chamber neck with SCL, number of eggs and clutch volume. Among female size parameters and clutch size parameters, significant correlations were observed between

BCI, and MFL. SCW, number of eggs, and clutch volume ($R^2 = 0.76, 0.43, 0.45$ and 0.35 , respectively, $P < 0.01$)

Preliminary comparisons of the recolonized and the natural beach by ANOVA showed no significant differences in individual body size, clutch size and nest site parameter means at the 2 locations. Application of Levene's F-test for homogeneity of variances showed several parameters to have unequal variances between the sites. Four nest site parameters - actual nest depth, chamber width, neck length, and air space - showed significant location-mediated inequality of variances ($P < 0.05$) (Table 4-2).

Among the nest site parameters, mean body pit depth and mean nest cavity depth did not differ significantly between sites, nor were their variances significantly different. However, it can readily be seen that although not nearly different in magnitude, the variances are skewed in distribution (Figs. 4-3 and 4-4). In both parameters, the observed range is shifted toward shallower depths in the recolonized beach. The variance in actual nest depth was significantly smaller at the recolonized beach ($P < 0.05$) (Fig. 4-3). The nest chamber measurements - width of chamber and length of neck - had variances which differed significantly by location ($P < 0.01$) (Figs. 4-5, 4-7). The width of the chamber is narrower and has a lower level of variance at the recolonized beach. Length of the neck neck behaved in the opposite manner, appearing longer with greater variance at the recolonized beach. Variances between the two locations for the ratio of neck cross-sectional area to chamber cross-sectional area were significantly different ($P < 0.01$). Significantly different variance is also seen in air space ($P < 0.05$) (Fig. 4-7), with greater variance at the recolonized site that is skewed toward less potential air space.

Discussion

Correlation of Burrow Size and Clutch Size Parameters with Nest Size

There are 3 general categories to which the significant size interactions in Table 4-1 can be assigned. In the first category, location helps to explain some of the variability in the data that can be explained without considering the difference in nesting media between the natural and reconstructed beaches. The relationships between MPL and neck width, SCL and neck width, SCW and nest volume, and SCW and surface fall into this group. In these correlations the medium confers advantages or disadvantages based on size, for example, greater MPL in the reconstructed site might produce a different amount of variation in neck width than in the natural site because of differential leverage in the denser medium. In the second category, factors other than location are important in defining the relationship and explaining variability. These include SCL with chamber length, SCW with chamber length and neck length, number of eggs with actual nest depth and chamber length, and clutch volume with actual nest depth, maximum egg depth, chamber length, and nest volume. These correlations perhaps give the most insight into some of the fundamental selective considerations in evolutionary determination of nest cavity size. In the third group, both location-specific effects and location-independent effects contribute significantly to explanation of the observed variability. The relationship of SCL with neck length, SCW with nest cavity depth, number of eggs with maximum egg depth and neck width, and clutch volume with neck width make up this latter group. These interactions

represent elements of nest-site determination that are not so evolutionarily canalized or mechanistically fixed as to have susceptibility to variability introduced by different media.

The small, but in some cases highly significant correlations between fluke size parameters and the nest-site parameters (Table 4-1) were a surprising and critical finding in this study. The rear flapper length of a nest turtle is believed to be an important factor in determining nest depth by its vertical insertion into the sand, and the rear end shape of the egg chamber by lateral rotational movements (Hallman and Ewins, 1992). The measurement of flapper length used in this study included the apical bones (ilia and ilia), the mesopodials (tarsi), the metapodials (metatarsi), and the phalanges. Due to difficulty of measurement, the femur was omitted, although its contribution to the limb's length and orientation cannot be ignored. Accepting proximal view to dorsal phalange as a valid measure of flapper length requires the assumption that for individual turtles, limb components scale continuously or at least proportionally. A higher level of plasticity in the development of the femur compared with other limb bones would preclude this but is unlikely given the similar constraints of mode of locomotion (swimming, walking, digging), resting, and range of motion under which they evolved (Jenkins, 1980; Raup, 1980). Gass *et al.* (1988) observed apical and lateral locomotion in leatherback turtles (*Dermochelys coriacea*), noting the similarity between swimming and walking movements, and the functioning of the hindlimb as a helical spade during digging. If specific selective pressures stemming from nest excavation alone were acting on the hindlimb then it is probable that effects would be most apparent in the distal portion of the

limb, the functions of swimming and walking likely place more balanced pressure on the entire limb (Bennett and Bell, 1997).

If limb length scales with body size, animals with greater SCL and SCW would be expected to have a longer MFL. Subsequent regression analysis of MFL with SCL and SCW gave R^2 values of 0.28 and 0.69 ($P < 0.01$), respectively, indicating that a substantial amount of the variation in flapper length is indeed related to body size. The act of digging the nest chamber to a suitable depth clearly requires a limb of a certain minimum length as a reproductively mature animal. Snail's-courtesy width was significantly correlated with nest entry depth, with and without consideration of location-specific effects (Table 4-4). This is probably due to the hardness of the medium and the extent to which it allows the animal's plastron or gird vertically to add length to the digging stroke, and the effect of body width on antero-lateral rear flapper reach. The new data indicate that small turtles (with shorter flappers) can and sometimes do dig deeper, larger nests than larger turtles. Part of this could be attributed to plastron width: the narrower body of a smaller animal might again permit more vertical rocking during the digging motions.

Beyond these considerations, regulation of nest chamber dimensions may have a component of behavioral control. Hulsman and Eklstrom (1997) reported the stimulus for termination of digging as the digging flapper coming up empty on at least 3 successive tries. Dool and Roberts (1991) found that while tactile stimulation of the flapper by nest dirt elicits nest depth, there is a strong non-behavioral component that readily overrides it. These findings are not mutually exclusive. They both involve perception of stimuli, one tactile, one temporal, and an appropriate behavioral response: cessation of digging. The

observation supports the hypothesis that nest chamber size may be partially regulated by the animal's hormone-mediated motivational state and individual behavioral fixed action patterns. This could be explored by testing successive nests of an individual at each of her nesting emergence over the course of a season and between nesting seasons, comparing the dimensions to see if there is digging the same shape of nest each time and if there is any evidence of increased flexibility of nest construction with experience, and monitoring of blood hormone levels during nesting.

The strongest relationship observed ($R^2=0.31$, $P<0.05$) was between number of eggs and maximum egg depth. The significance of the "without location effects" R^2 values for number of eggs and depth volume suggests that these parameters exert a selective pressure affecting the use of the chamber constructed by a female. Clearly selective pressure acts in the evolution of sea turtle nesting behavior would have favored survival of nesting habits which allowed for complete egg coverage. The construction a turtle makes in use of her offspring by preparation of the nest chamber appears to be at least partially dependent on her body size and reproductive output per nesting event.

Comparison of Clutch Size and Nest Size Parameters Between the Natural Beach and the Encouraged Beach

Although the means for all the parameters measured did not differ significantly between the natural and encouraged locations, the variances tended to be significantly different, most striking. The skewed variances seen in body pit depth and nest cavity depth at the encouraged beach (Figs. 4-3, 4-6) can be directly attributed to the differences

between the natural and reconstructed beaches. This is reinforced by the significantly divergent variation in actual nest depth (Fig. 4-3). The natural beach and the reconstructed beach were visually different. The sand at the reconstructed beach was darker and finer grained, and there was a definite denser, coarser, shell-filled layer at 33-60 cm deep (Fig. 4-6a). This layer may have mechanically constrained the animal's ability to dig and buffered the variance at the reconstructed beach. The significantly different variances in width of the chamber and length of the chamber neck (Table 4-2)-(Fig. 4-6b) indicate that the difference among media exerted an effect on the turtle's digging motions. Both of these measurements were taken horizontally through the nest and reflect the lateral range of motion of both the proximal and distal rear flippers. The fact that length of the chamber neck at the reconstructed site shows greater variability than at the natural beach, whereas width of the chamber bottom at the reconstructed site shows less variability (Figs. 4-6, 4-7) implies that there is some mechanical advantage or disadvantage being conferred by the medium in different sections of the digging sequence and flipper strokes. Extensive subsequent analyses comparing differences and series of cross-sectional areas of necks and chambers between the two beaches yielded no significant results. It appears that the constraints the medium places on the kinematics of the hindlimbs act to reshape rather than constrain the parts of the nest cavity. The net result is a tendency for nests at the reconstructed beach to have walls that are closer to vertical than those at the natural beach. Nelson *et al.* (1987) observed tapering of nest chambers dug in hard-packed sands similar to that seen at the reconstructed beach and postulated a reduction in chamber volume as a result. The nest volumes seen in the current study did not reflect such a reduction, but

were somewhat more variable in the denser mud of the reconstructed beach. Montague (1973) cited the importance of avoiding nests that form concavities in surrounding materials because of their potential to shear the excavation surfaces of the nesting female.

The variance in air space in the chambers of the reconstructed beach was significantly greater than that of the natural beach ($P=0.012$) but that variance is directed towards less air space (Fig. 4-4). This is a natural consequence of the altered nest geometry seen at the reconstructed site. In the nests from the natural beach, with their variances directed toward larger (horizontally), narrower nests, the first sand clumped to by the female upon arriving clumps on the ventral, top eggs, and the sand that follows proceeds to plug the neck of the cavity. This permits persistence of lateral and over-egg air spaces. In the nests from the reconstructed beach with wider necks, covering sand obliterated potential lateral air spaces, and tends to obliterate the over-egg air spaces to a greater degree. The presence of air space in the nest chamber is a biologically important phenomenon. Air spaces established when nest construction and evaporation are completed permit through circulation, allowing expansion of the eggs as they take up water, controlling the pressure and hydrostatic volume around each egg and allowing hatchlings room to wriggle as they pop and emerge. The depth of the nest chamber, and the structure of the nestbank affect gas exchange between the chamber and the atmosphere (Asherman, 1973), so air space within the nest may also serve as a reservoir and to sustain a stronger diffusion gradient for moving of gases. Kato (1973) noted that despite adaptations to ventilated habitats, *Circulicorella carolinensis* embryos can only maintain normal levels of oxygen consumption if the critical oxygen tension within the nest remains high.

Embryonic requirements for oxygen become greater towards the latter part of incubation (Ellenbogen, 1949; Maloney *et al.* 1978) making permeation of the air space all the more critical. Unlike the chicken where eggs that do not appear to benefit greatly from small modifications in humidity (Wachling, 1982), sea turtle embryos may depend on the water vapor present in the chamber air space to maintain hydration of some of the eggs.

These insights about the steps that a clutch of eggs must endure as a result of chamber steps naturally lead to questions about the types of sub-environments imposed on the eggs. Chapter 3 addresses this by examining the effect of egg position within the nest on development and hatching.

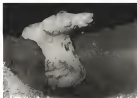


Figure 4-1 Nest cavity cross: a) Cut from top of eggs to beach/body pit surface (not provided for scale) b) Partially excavated part of tunnel chamber. Turtle was facing left edge of photo

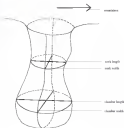


Figure 4-2. Diagram of nest case, showing location of measurements taken (neck width, neck length, chamber width, chamber length) and orientation of nesting logarithm (*C. carolinensis*).

Table 4-1 Results of correlation analyses (R^2 values) of female body size parameters and clutch size parameters with nest size parameters. The upper value for each correlation is an overall R^2 and the bottom value reflects removal of location specific effects ($\eta^2 = P=0.05$).

	Mean Flipper Length	Snigle Carapace Length	Snigle Carapace Width	Number of Eggs	Mean Egg Volume	Clutch Volume
body gtl	0.01	0.06	0.07	0.06	0.07	0.04
depth	0.02	0.05	0.01	0.00	0.00	0.00
nest cavity depth	0.13	0.14	0.20 *	0.13	0.11	0.13
	0.02	0.02	0.10 *	0.06	0.02	0.08
actual wet depth	0.03	0.04	0.10	0.13	0.02	0.13
	0.01	0.02	0.06	0.11 *	0.01	0.12 *
maximum egg depth	0.02	0.10	0.04	0.21 *	0.05	0.14
	0.01	0.01	0.00	0.09 *	0.01	0.10 *
chamber length	0.13	0.13	0.16	0.34	0.04	0.13
	0.01	0.12 *	0.12 *	0.11 *	0.02	0.10 *
chamber width	0.08	0.02	0.04	0.07	0.12	0.04
	0.01	0.01	0.00	0.02	0.06	0.00
neck length	0.02	0.10 *	0.11	0.06	0.04	0.00
	0.06	0.14 *	0.09 *	0.04	0.01	0.02
neck width	0.10 *	0.20 *	0.11	0.26 *	0.08	0.20 *
	0.00	0.06	0.00	0.12 *	0.02	0.16 *
neck volume	0.02	0.30	0.01	0.02	0.00	0.00
	0.02	0.08	0.01	0.02	0.02	0.00
nest volume	0.04	0.12	0.26 *	0.13	0.20	0.20
	0.01	0.11	0.08	0.02	0.09	0.13 *
air space	0.04	0.10	0.20 *	0.06	0.00	0.00
	0.06	0.06	0.01	0.02	0.00	0.00

Table 4-2. Least-squared means (LSM) for body size, clutch size and nest chamber size parameters and results of Levene's F-test for homogeneity of variances between the natural beach and the regenerated beach. All linear measurements are in cm and all volume measurements are given in ml

Variable	LSM at Natural Beach \pm SE (n)	LSM at Regenerated Beach \pm SE (n)	Levene's F ($\alpha = 0.05$)
mean dipper length	21.6 \pm 0.3 (35)	21.3 \pm 0.3 (33)	0.89
straight culmen length	50.4 \pm 0.2 (35)	50.3 \pm 0.3 (33)	0.32
wing to culmen width	85.5 \pm 0.4 (35)	71.3 \pm 0.4 (33)	0.33
number of eggs	102.8 \pm 0.3 (35)	104.5 \pm 0.2 (34)	0.86
mean egg volume	38.6 \pm 0.3 (35)	35.3 \pm 0.3 (33)	0.97
clutch volume	4015.6 \pm 144.9 (35)	3676.3 \pm 221.5 (34)	0.83
body pt. depth	20.6 \pm 0.3 (35)	18.5 \pm 0.4 (33)	0.87
nest cavity depth	99.3 \pm 1.8 (35)	88.6 \pm 0.9 (33)	0.57
actual nest depth	38.6 \pm 0.8 (35)	37.6 \pm 0.3 (33)	0.88
maximum egg depth	34.6 \pm 0.2 (35)	39.3 \pm 0.8 (33)	0.43
chamber length	21.4 \pm 0.3 (35)	24.8 \pm 0.8 (33)	0.54
chamber width	21.7 \pm 0.2 (35)	22.4 \pm 0.3 (33)	0.33
neck length	20.5 \pm 0.3 (35)	20.8 \pm 0.8 (33)	0.88
neck width	66.3 \pm 0.4 (35)	44.3 \pm 0.4 (33)	0.26
neck volume	1536.3 \pm 215.8 (35)	3071.4 \pm 258.7 (33)	0.66
entr. volume	34026.9 \pm 418.3 (33)	10867.5 \pm 356.6 (33)	0.13
air space	3240.6 \pm 307.8 (35)	3480.2 \pm 343.4 (33)	0.93

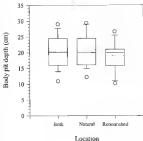


Figure 4.3: Observed values for body pit depth of loggerhead turtles (*C. carolinensis*) at a natural and a restored beach. Values are combined as 'spots'.

dotted line - mean

solid line - median

box - 10% of observed values

whiskers - 95% of observed values

outliers - 5% of observed values

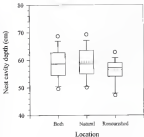


Figure 4-4: Observed values for nest cavity depth of loggerhead turtles (*C. caretta*) at a natural and a reconstructed beach. Values are combined as "both".

dashed line : mean

solid line : median

box : 50% of observed values

upper box : 80% of observed values

outliers : 10% of observed values

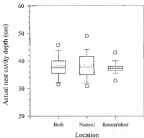


Figure 4-6. Observed values for actual nest cavity depth of logskipped turtles (*C. serrata*) at a natural and a reconstructed beach. Values are combined in 'both'.

dotted line - mean

solid line - median

box - 50% of observed values

error bars - 80% of observed values

outliers - 90% of observed values

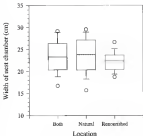


Figure 4-6 Observed values for width of chamber of nests dug by loggerhead turtles (*C. caretta*) at a natural and a recolonized beach. Values are reported in "both".

dotted line - mean

solid line - median

box - 50% of observed values

error bars - 80% of observed values

outliers - 90% of observed values

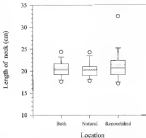


Figure 4.7 Observed values for length of neck (horizontal) of non-random dug by loggishorn tortoise (*G. carolinensis*) at a natural and a remounted beach. Values are combined in 'bath'.

dotted line = mean

solid line = median

box = 50% of observed values

error bars = 90% of observed values

outliers = 95% of observed values

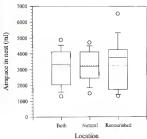


Figure 4-8: Observed values for potential free air space in nests dug by loggerhead turtles (*C. caretta*) at a natural and a recolonised beach. Values are combined as 'pairs'.

dotted line - mean

solid line - median

box - 50% of observed values

whiskers - 80% of observed values

outliers - 80% of observed values



Figure 4-9. Non-pitted casts: a) Ventral view of casts from encrusted site (top left) and natural site, with microliths raising major showing difference is particularly seen at root bottom. b) Left and control casts are from the natural beach, right cast is from the encrusted beach.



Figure 6-13 Anterior view of nest cavity (a) (from the a) matured and b) resounded brood after. Note relative sizes of neck and egg chamber on each.

CHAPTER 5
EGG AND HATCHLING RESPONSES TO NEST POSITION IN NESTS OF
LOGGERHEAD TURTLES (*Caretta caretta*)

Introduction

The effect of the nesting site selected by a sea turtle on the subsequent development of the deposited clutch is dictated on a gross level by parameters such as location on the beach, sand quality, presence or absence of shade, and weather. The shape and depth of the nest chamber influence how these external factors will affect each egg. Once an egg is dropped into the chamber, it remains in one position throughout the entire incubation period as a result of its contact points with sand, air, and other eggs. This study links the work described in Chapters 3 and 4, first by defining the extent to which variation in embryonic development is affected by the incubation environment, and secondly by examining the consequences of the maternal investment in placing and preparing the nest.

The effects of egg position within the nest on developmental aspects of reptile embryos and hatchlings have not received a lot of attention from researchers. The relative ease of controlling the laboratory environment has yielded many studies of eggs incubated in isolation; these provide valuable information but may not reflect responses of eggs in their natural, grouped environment. Ackerman (1980) indicated the importance of egg

position within the nest as the result of one factor: egg temperature in the nest. A nest water's cooling behavior results in a clutch that is surrounded by, but not packed in, water. This gives the nest a 5-15 fold partial pressure gradient center to edge rather than one 2-3 times greater. Consequences of the latter would include slower growth, later hatching and increased mortality for inner eggs.

Response to hyaline conditions is also contingent on position in the nest. Hocking *et al.* (1987) demonstrated that egg position can significantly affect egg mass and hatching mass within wrapping turtle nests through relative availability of water. As plastic-shelled reptile eggs go through incubation they lose and gain water, becoming flaccid or turgid depending on conditions. Egg density (mass/volume) can provide an good indication of water content in an egg relative to its volume. Harrison and Blount (1991) compared water densities for different types of reptile eggs; however, actual changes in egg density over the course of incubation and by position have not been described. Much work has been done on the water exchange relationships in reptile nests (Pickard *et al.*, 1982; Thompson, 1987) that determine the density change.

The nest position that an egg holds throughout incubation may have additional developmental consequences for the embryo. As reptile embryos continue to develop after egg deposition, the shell layers dry, and formation of the chorionallantois separates in the inner shell causes the appearance of banding, which spreads over the shell as the membranes develop (Thompson, 1987). This "banding" is a developmental function initiated within the egg, and position within the nest would not be expected to affect it. Observation of differential banding between air-incubated and water-incubated eggs is

Chapter 5, however, raises the question of whether there are maternal influences affecting formation of this important gas, solute and water exchange membrane. Length of incubation period and hatching synchrony may also be affected by parent-related macroclimate (McCluskey, 1975; Mironovskiy, 1980; Mironovskiy and Yutkina, 1982). Metabolic heat from the clutch mass has the potential to influence developmental rate of the embryo. If environmental conditions place a clutch at the pivotal temperature during the critical period for sex determination, metabolic heat can produce females from control incubated eggs and males from peripheral eggs (Blomden *et al.*, 1994).

Chapters 2 and 3 discussed the eggshell and how changes in its structure facilitate the incubation process. Based on the differential dissolution of calcium carbonate observed for eggshells of some clutches in sand and in media, the macroclimate found in different nest positions may cause similar responses.

Egg mass, density and hatching are important considerations of the hyaline and developmental quality of an incubating egg. Incubation duration, hatching mass, sex and eggshell calcium content address hatching quality and potential contribution to the population. The female surface goes where these critical aspects are being cultivated: she invests a particular kind of hole to safeguard her reproductive investment. This study examined the effects of egg position within natural nests on these characteristics of embryo physiology and development, with a view toward understanding the environment a female prepares for her eggs by digging a nest.

Methods

Nests of 48 Hopperband turtles (*Caretta caretta*) were marked at Melbourne Beach, Florida, and scheduled for individual examination at 1 of 8 times during the incubation period. Five nests were examined at each of 8 times (0, 3, 5, 10, 15, 30, 45 days, and hatching minus ~2 days). Immediately after oviposition, each nest was marked with 3 stakes: 1 in the vegetation line, 1 in the dense vegetation aligned with the nest and 0.3 m behind the first stake, and the third 1 m south of the first stake. Accurate measurements from all stakes to the nest site were taken, and an aluminum can was buried 0.5 m south of the nest. This marking system enabled accurate relocation of the nests for examination. Orientation of the turtle on the nest was noted. On completion of laying, the female was checked for tags, and straight compass length (SCL: nuchal notch to pygal notch) and straight compass width (SCW: taken dorsally from nuchal point of compass) were measured with calipers.

At the scheduled time, a pit 1 m deep was dug lateral to each nest (based on the animal's nesting orientation) (Fig. 3-14). The nest chamber was carefully exposed from the top and side to allow removal and scoring of eggs by their position within the nest. Eggs appressed to the nest and in contact with sand in the neck region of the nest cavity were designated as top eggs; those buried in the cavity and in contact with the floor of the nest chamber were designated as bottom eggs. Eggs between the top and bottom and contacting the sand or air pockets at the nest periphery were classified as side eggs.

These eggs at the center of the clutch with other eggs well into egg or again as their only contacts were classified as inner eggs (Fig. 3-1b). After all eggs were removed and sorted 10 eggs from each of the 4 areas within the nest were chosen at random to be weighed to the nearest 0.01 g on a portable electronic balance. These eggs were also measured for maximum and minimum diameter to the nearest 0.01 mm with floorplan-dial calipers. Banding of one turtle-egg progresses from the top down to the bottom: the diameter at the site of banding was measured, and banding was recorded as the percentage of that egg diameter which exhibited narrowing and loss of translucence. Egg volume was calculated as an ellipsoid ($V = 4/3\pi ab^2$, where a = maximum, b = minimum). Egg density was calculated by dividing mass by volume. The total number of eggs from each position was recorded for all of the clutches excavated. All eggs, except those excavated near full-term, were carefully re-banded with their original orientation maintained. Measured eggs from nests excavated 2-3 days prior to hatching were isolated and incubated in the laboratory until hatching. At peeping time of peeping recorded as measurement DATE, a sample of chorionallantoic fluid was taken to determine sex by calculating an estradiol/testosterone (E/T) ratio following quantification of these hormones in the chorionallantoic fluid using validated radioimmunoassays (Green *et al.*, 1993). An E/T ratio of 1.00 was used as the determinate index value. The hatching was weighed on emergence from the egg. Eggs from 3 additional clutches were excavated and sorted by position 2 days prior to hatching. After hatching, the shells of all viable eggs were analyzed for calcium content by gas flame atomic absorption spectrophotometry (see Chap. 3 methods).

The relationship between clutch size and proportion of eggs at each nest position was examined using SAS PROC REG (SAS Institute, 1988). SAS PROC MIXED (Sokal and Rohlf, 1995) was employed to examine the effects of clutch, age and egg position in the nest on mass, frequency and banding with a mixed ANOVA. Effects of egg position and clutch on incubation duration, hatching mass, hatching sex and eggshell calcium content were analysed using SAS PROC GLM (SAS Institute, 1988) for standard ANOVA.

Results

The sizes of the clutches examined for this study ranged from 47 to 103 eggs. Table 1-1 summarises the numbers of eggs at the 4 different positions within the nest. Generally a clutch contains between 1/4 and 1/3 nest eggs. Negative correlations were found between clutch size and the proportions of top and bottom eggs in a clutch ($P < 0.05$) (Fig. 1-2, 1-3). No relationship between clutch size and proportion of side eggs was observed ($R^2 = 0.001$, $P < 0.05$) (Fig. 1-4). Proportions of nest eggs increased with increasing clutch size ($P < 0.01$) (Fig. 1-5).

The effects of position within the nest on egg mass are shown in Figure 1-6. Only top eggs changed significantly in mass from time of deposition to hatching ($P < 0.05$), increasing sharply between 45 days and hatching. There is a non-significant tendency for eggs at all positions to decrease in mass between deposition and day 5. Mean egg mass did not differ significantly among positions up to 45 days of incubation. Immediately prior

to hatching, mean egg masses at all nest positions differ significantly from each other ($P < 0.05$) except for bottom and rear eggs.

Density of rear eggs changed significantly over the incubation period (Fig. 5-7). Top eggs on day 10 were more dense than rear eggs ($P < 0.05$). On days 15 and 20 rear eggs were less dense than bottom eggs, and by day 45 rear eggs remained less dense than eggs at all other positions until hatching. Banding did not vary significantly with position (Fig. 5-8), although a tendency toward slightly less banding was observed in the top eggs.

Examination of egg position-effects on duration of incubation, and hatching mass and sex revealed highly significant interactions between clutch and position ($P < 0.05$), along with simple effects. The data were sorted by clutch to allow examination of the simple effects of egg position for the 5 full term clutches.

For duration of incubation, clutches 24, 25 and 264 showed significant position effects ($P < 0.05$) (Fig. 5-9). Top eggs in clutch 24 pipped significantly earlier than those from other positions. In clutches 25 and 264, bottom eggs took longer to pip than eggs from other positions. Generally, eggs in each clutch pipped within 3 days of each other. With the exception of rear eggs from clutch 21, clutches 24 and 25 took but significantly longer incubation times than the other 3 clutches.

Significant effects of position on hatching mass were found, with top hatchlings weighing less ($P < 0.05$) than those at all other positions in 4 of the 5 clutches (Fig. 5-10). Bottom hatchlings were significantly heavier than side and rear hatchlings in clutches 264 and 230 and showed a tendency to be heavier in clutch 24 and 283. Side and rear hatching masses were similar within individual clutches.

The effect of clutch on the hatching sex by position analysis was great, as can be seen by the widely differing results for each (Fig. 3-11). Three of the 5 clutches showed non-significant effects of egg position on hatching sex. Significant effects were seen in clutch 34 where top eggs yielded a lower proportion of females, and in clutch 124 where inner eggs produced more females than did bottom and top eggs.

No significant effect of egg position in the nest on eggshell-calcium content was observed ($P = 0.05$) (Fig. 3-12). Trends in clutches 3 and 5 were similar, with top and inner eggs tending to retain more of the applied calcium while bottom eggs lost the most. Clutch 4 showed the reverse, with side and bottom eggs retaining more calcium.

Discussion

Egg-Position Determinants and Characterization of Distribution

The distribution of eggs by position in a particular sex tortle nest is determined by 3 major factors: the size and shape of the nest chamber dug by the female, and the number of eggs she places within that chamber. These components interact dynamically to produce the observed allocation of eggs. If a large chamber is only half-filled with eggs, eggs being covered it will have proportionally many more eggs in peripheral contact with the sand than if it had been completely filled. This will occur at the expense of inner egg numbers and/or space.

The negative correlations of proportions of top and bottom eggs with clutch size (Figs. 3-3, 3-5) are readily explained by the shape of the nest cavity. The upper and lower walls of the cavity taper; only a limited number of eggs can fall into bottom positions before they are covered by others. In subsequent laying, a point is reached when, increasing the number of eggs, nest fills the central horizontal block of the nest. Then, falling eggs may pile up in the center or roll off to the sides to fill the shoulders of the cavity. In either case, the proportion of top eggs is determined at laying of those last eggs and covering of the cavity. The greater slope of the top egg correlation (Fig. 3-3) indicates that the proportion of top eggs decreases more rapidly as clutch size increases - this is probably a result of filling the rounded neck of the chamber. Lack of a significant correlation between proportion of side eggs and clutch size again reflects the boundaries set by the nest chamber walls, cavity shape and filling method: there are only a limited number of side positions but how quickly they become occupied in the central block of the nest, like a relatively independent of the number of eggs added. Proportion of inner eggs (Fig. 3-5) is mediated by the size and shape of the chamber and how completely the female fills a nest eggs. Larger clutch sizes increase the likelihood that eggs will fill all peripheral positions and be available to support inner eggs.

In their analysis of egg positions in 50 snapping turtle nests, *Frederick et al.* (2013) found 94% of the 2500 eggs surveyed to be in contact with the mother. The range of clutch sizes in the data reported was 13-44 eggs. They attributed this large percentage of maternal contact to high incidence of nest chamber collapse due to loose, sandy nesting medium, and obstruction of proper digging by rocks and roots. The latter

incubation was observed to produce compressed chambers with insufficient internal space to allow eggs to be easily supported by other eggs, there was noted as a departure from the "typical flask shape" (B). Both collapse and compression of nest chambers limit the amount of air space that is vital for gas-exchange, as discussed in Chapter 4. Logghead nests may collapse due to excessive drying of the medium, incubation and vibrations, or poor construction because of spaced layers or digging obstructions.

A typical logghead nest will have 75% of its eggs in peripheral positions within the clutch (Table 1). The top and bottom egg components of this number are in contact with the incubation medium, with egg contact with the medium in the usually observed case, but can be contingent on laying and chamber filling mechanics. This allocation is a compromise between maintaining egg-medium contact, which enhances water uptake and thereby hatching quality (Caughey and Collins, 1993) and spacing the clutch with more eggs to allow needed air space.

Effect of Incubation on Egg Parameters

Almost all of the changes seen in reptilian egg masses over incubation are due to water exchange (Mack, 1993). The observed fluctuations in egg mass (Fig. 5-6) were less pronounced than the steady declines reported for flexible-shelled nesting birds (*Chelidony argentea*) eggs incubated in relatively clear media (Munn, et al., 1981). The initial decrease in egg mass observed in the first few days of incubation probably represents a period of equilibration with the chamber environment. Tracy and Smith (1983) reported net losses in mass of logghead eggs over incubation, in the range of -8

to +10% of initial egg mass. The top eggs in the current study actually gained 12-13% in mass. Week (1980) observed that any change in the mass of incubating eggs is due to hyaline fluid. Therefore, contributions by precipitation, condensation and groundwater to moisture in the medium would have been the possible sources for the change. No data on groundwater levels at the study site were collected. The significant increases observed in mean mass of top eggs before hatching was possibly due to their proximity to the surface of the nest. This would have increased the impact of the afternoon precipitation that was prevalent during the period when these eggs were collected. Ackerman (1981) and Week (1980) note the equality with which water uptake can occur in double-shelled eggs; this may be further accentuated closer to hatching, when high solute levels in the egg fluids can create large osmotic gradients with any water available in the environment. Considerable amounts of precipitation are needed to wet the beach through to nest depth; many of the quick rainfall events only wet the first 2-4 cm. Water percolating down through the medium would first reach top eggs, and if they are in a water deprived state, may be completely absorbed at that level.

When sea turtle eggs are first laid they are quite variable in their water content, ranging from extremely wet (4) to dried. Week (1980) notes similar variation in mudflat and beach eggs of various species of turtles and lizards. Palmer and Gaulton (1988) discussed the addition of "plumping water" to the albumen of *Cryptotriton polyphemus* eggs in water. The variation observed in sea turtle eggs possibly reflects random water within the female and her level of tissue hydration. As with mass, any increase in turgidity of sea turtle eggs has to come from water taken up from the incubation medium. Difficulties

incubated in measuring rigidity led to the use of density as an indicator of the water balance within an egg. This is based on water having a higher density than the yolk, most of the egg.

Pedward *et al.* (1980) and Bonting *et al.* (1983) noted that eggs suspended in the center of a clutch may be exposed to a different hyaline regime than eggs in contact with the medium. This is corroborated by the significant differences seen in density (Fig. 3.7). Bottom and top eggs maintained the highest mean levels of density throughout incubation. The lower level of rigidity seen in side eggs is likely due to peripheral air space contact rather than sand contact in many of the eggs. Because of the manner in which a nest is filled and covered by the female turtle, only bottom and top eggs are ensured of having contact with the medium; side eggs near the bottom are likely to have contact, whereas side eggs near the top are most likely to be bordered by airspace. In the laboratory incubation experiment discussed in Chapter 5, eggs completely buried in sand were hatched throughout incubation. Despite the high humidity maintained, the air incubated eggs lost water and developed abnormalities. Peripheral contact with the incubation medium clearly plays an important role in maintaining egg hydration. The mud turtles (*Chelodactylus flaviventris*) release urine to wet the soil during oviposition (Iverson, 1985), but sea turtles, nesting from a hypertonic medium where internal water is at a premium, have not been observed to use this strategy. The moisture that is deposited in varying amounts along with the eggs during laying may help to limit sand water loss.

Differences in percentage of hatching were not significant among different egg positions in the nest. The naturally variable ages of hatching indicate drying of the

eggshell and thickness of the chorioallantoic membrane (Thompson, 1981). Semi-incubated eggs from the Chapter 3 study showed 68-70% banding whereas air incubated eggs banded completely. Some top eggs in the current study showed uneven banding or a lower amount of banding. In both cases, ready availability of external water could account for the appearance of banding or drying. Kam (1993a) compared the amount of vascularization in the chorioallantoic membranes of normoxic and hypoxic eggs of the Florida red-bellied turtle (*Pseudemys floridana*) and found a significant increase in the length/density of blood vessels in hypoxic eggs due to banding. While banding as measured in the current study (percentage of external appearance) did not reveal any positional differences, future examination of membrane vasculature of eggs from different locations in the nest could elucidate subtle developmental responses to partial macrochlamia.

Effects of Egg Position on Hatching Parameters

The varying results observed in the examination of the effects of egg position on incubation duration emphasize the over-riding importance of nest placement by the female. Incubation length is inversely correlated with temperature (McClintock, 1972; Mrosovsky, 1982). Therefore, variations in the mean incubation temperature at different nest positions can result or accentuate egg development. Temperature differences between top and bottom eggs in the nests of pig-nosed turtles (*Carettochelys insculpta*) were found to vary by as much as 3.5° C, based on banding by incubation. Hatch 24 may have reached extended incubation because of its central location in the track, away from the low-dense

and not subject to much shading. This may have caused the top eggs to develop faster and pop sooner than deeper eggs that are more greatly influenced by the thermal regime generally found at near depths (Ikonomov, 1982). Clutches in which the bottom eggs pipped significantly later (clutches 23 and 26) were likely exposed to groundwater effects such as cooling or uneven gas transport, and also may not have received the benefit of top metabolic heating. McGeehan (1990) reported longer incubation times for loggerhead eggs artificially incubated in water media, citing gas diffusion limitations in the water medium as a possible cause.

Eggs in the current study were taken into a stable thermal environment 2-3 days before proposed hatch date and all the viable eggs in each clutch pipped within 3 days of each other. Hays et al. (1982) cite an extreme case in which hatchlings from a single nest emerged at the sand surface over the course of 15 days, which suggests that pipping took place over a longer interval. Factors such as gas exchange may interact with temperature to produce the observed range of effect on duration of incubation (Asherson, 1982). Hydric conditions can influence the metabolism of avian embryos to produce larger hatchlings (Yock, 1970; Packard, 1994). However, at least in some freshwater turtles (*Trachemys scripta*) water does not affect critical oxygen tension and yolk bio-hatching mass (Karr and Lohmeyer, 1994). Therefore, hydric effects on incubation duration may be limited to moderating temperature changes, or the effects of severe flooding.

Usually greater water availability is positively correlated with greater hatching mass in double-shelled eggs (Morris et al., 1993; Hocking et al., 1993; Thompson, 1997), based on more complete utilization of yolk mass. The top eggs in this study differed in

their response by producing lighter hatchlings, although they were in contact with the medium. As Figure 5-4 shows, top eggs did not differ significantly in mass (and therefore in water content) until just a few days prior to hatching. The very late increase in water availability may have prevented it from being channelled into yolk lipid metabolism and augmentation of hatching mass. Gellinger et al. (1984) found that late embryonic snapping turtles (*Chelydra serpentina*) incubated under water conditions attained significantly higher body masses than those incubated under other conditions. Bottom hatchlings in the current study were significantly heavier in 2 clutches and showed a trend toward greater mass in 1 other. The bottom of the nest chamber appears to provide a very favorable hydric environment during incubation.

Hatching sex is primarily affected by nest placement around like incubation duration, is temperature dependent. The sex ratio in a nest will be more profoundly affected by constant direct incubation throughout the incubation period, than by routine temperature differences due to metabolic heating within a clutch. The preponderance of a single sex within clutches has been documented for loggerhead nests in Florida (Mironovsky and Fraenkel, 1989), so the sex-significant effect of position in 3 of the 5 clutches was not surprising (Fig. 5-11). Metabolic heating within a clutch can affect the sex ratio (Mironovsky and Tishina, 1982), especially if the clutch is within the transitional temperature range for sex determination. In clutch 204 the lower eggs produced all females, whereas top and bottom eggs yielded 20-30% females. Side eggs from this clutch produced 60% females. This pattern of sex ratios suggests that metabolic heating played a major role in sex determination within this clutch. Georgia (1992) described

thermal gradient is series of pig roost boxes (*Taeniopygia castaneiceps*) that resulted in deeper - cooler eggs producing males and upper - warmer eggs producing females. Members of this species can produce both sexes by either being at the proximal temperature or maintaining thermal gradient based on incubation. Sex ratio seems appear to have similar potential.

Effects of Position on Eggshell Calcium Content:

As discussed in Chapter 3, eggshell-calcium-mobilization depends on the hydro- and gaseous environment surrounding each egg. Unlike the significant differences seen with the incubation-experiments of air and sand in the laboratory, the differences among egg positions in spent eggshell calcium content were not significant. This reflects the mobility of the eggs-clusters in the nest. Top and bottom eggs are in definite medium contact and side-eggs may have some contact. Inner eggs may have ready access to gases within the nest chamber, but depend on capillary action or very high humidity for their water (Pohlert-et al., 1981). The similar trends seen in chapters 3 and 4 (Fig. 3-12) have inner eggs paralleling the air-incubated eggs in the lab by retaining more-calcium and bottom eggs being more calcium in a manner comparable to the sand incubated-eggs. The high calcium retention of the top eggs raises questions about the hydration levels of the sand over those particular nests and the gas circulation within them. It is not known whether carbon dioxide accumulates in particular areas of a natural nest chamber, but a pooling of carbon dioxide in the lower chamber combined with dry sand on top could account for the calcium levels observed in top eggs. A larger sampling of natural nest chambers might

find more in which positional effects are significant, precisely because of the range of variation in beach conditions.

Conclusions

Of the parameters examined in this study, egg mass and fertility showed responses that could be directly attributed to egg position within the nest. These factors are mediated predominantly by the shape of the chamber the female digs. Incubation duration, hatching mass and hatching sex respond to clutch effects as well as position effects. The control exercised by the female in this case is on a larger scale, involving chamber shape, depth, and placement on the beach relative to sandstone and water availability.

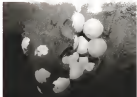
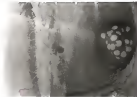


Figure 3-1. Nest excavation. (a) Clutch of eggs exposed from top and side as preparation for sorting of eggs by position. (b) Clutch with several eggs moved, more eggs and air spaces are visible.

Table 3-1 Number of eggs classified in each position for 10 sequential logarithmic ratio clutches. Totals by position for all clutches are given, as well as per clutch means, percentage and standard error. Minimum and maximum counts observed at each position are also shown

	Top	Sub	Bottom	Inner	Total
Total	737	1872	615	1242	4476
Mean	74	48	15	31	178
% of Total	16	42	14	28	100
SE	0.71	1.67	0.68	1.63	1.78
Minimum	10	28	3	11	61
Maximum	31	69	28	74	202

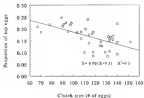


Figure 3-2. Effect of clutch size on the proportion of eggs observed at top positions within test chambers of loggerhead turtles ($P < 0.05$).

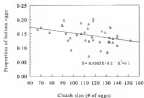


Figure 1-3 Effect of clutch size on the proportion of eggs observed in bottom position within nest chambers of logperch larvae ($P=0.05$)

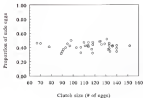


Figure 3-4 Effect of clutch size on the proportion of eggs observed in side positions within nest chambers of loggerhead turtles

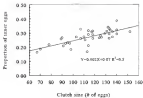


Figure 3-2 Effect of chord size on the proportion of eggs observed at user positions within user clusters of logarithmic order ($P=0.01$)

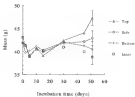


Figure 3-4 Mean egg mass at 4 positions within the clutch during incubation. Data are from 40 baggishawl turtle clutches from incubator 2B.

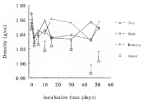


Figure 5.7 Mean egg density at 4 positions within the clutch during incubation. Data are from 40 logarithmic weight clutches (Barry et al. 2002)

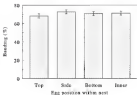


Figure 3-8 Mass percentage of banding observed from eggs at 4 positions within the clutch during incubation. Data are from 40 eggshells per clutch cluster. Error indicates SE.

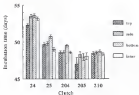


Figure 3-5 Incubation times for eggs at 4 positions within loggisholm nests during incubation. Data shown are means from 5 clutches. Bars indicate 2 SE.

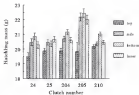


Figure 3-10 Mass of hatchlings from eggs at 4 positions within loggishand nests. Data shown are means from 3 clutches. Bars indicate SE.

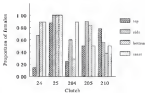


Figure 3-11 Proportion of females hatched from eggs at 4 positions within 3 logarithmic weeks

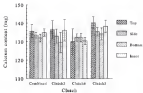


Figure 3-12. Calcium content of shells from hatched eggs at 4 positions within logarithmic nests. Data shown are means from 3 clutches. Bars indicate SE.

CHAPTER 4 DISCUSSION AND CONCLUSIONS

The Eggshell as Parental Investment in Care

The results of the scanning electron microscopy investigations, the laboratory incubations of eggs in air and acid, and the calcium analyses of eggs incubated *in vitro* provide strong support for consideration of the eggshell as an important component of parental investment in the egg. Despite the lack of any effect of egg position within the nest on calcium dissolution (Chapter 3), the depleted eggshells showed a loss of calcium similar in magnitude to the nest incubated eggs (Figs. 3-3, 3-10). The quantity of calcium carbonate deposited on the surface of the shell far exceeds the amount needed to produce a viable hatchling. The fact that the calcareous layer persists through most of the incubation is to be expected by the carbon dioxide build-up from the pre-hatching and the subsequent metabolic acid pulse. Indicates that the eggshell mineral layer is a responsive system that generally has sufficient thickness and density to perform all its roles. This observation is also supported by the weakening of the basal layer of calcium carbonate closest to the chorion membrane (as observed in Chapter 3) which may facilitate entry of the membrane by the hatching's egg tooth and subsequent pipping.

The fresh yolk of the sea turtle eggs did not differ significantly among clutches or initial calcium content (P=0.85) (Table 3-1) whereas the initial calcium content of fresh eggshell did vary (Fig. 3-2). The greater variation in calcium content of the eggshell perhaps reflects the more substantial range of variability to which the proposed aspect of parental investment responds. Selection pressures affecting investment of resources in the yolk such as parental energetic and spatial egg site considerations (Compton and Gilmore, 1990) can remain fairly stable over long periods of time for sea turtles. The magnitude and type of selection pressures that affect eggshell thickness vary with every nest deposited on the beach. Kaplan and Cooper (1984) proposed that natural selection may limit developmental plasticity in conditions of environmental instability, resulting in a range of observed characters, that was put forth as an alternative to optimal egg size theory but can also explain the observed range of investment in eggshell calcium.

The Nest Chamber as Parental Investment in Care

Parkard and Parkard (1986) cited an 11-16% decline in egg mass due to water loss in coastal eggs of snapping turtle nests as being similar to 15-18% expected for marine eggs. The water loss from lesser eggs in the current study was in the range of 9-11%. These ranges are all consistent with the type of incubation environment and which the respective eggs are placed, and their relative reliance on external sources of water for development. Arid eggs are the positional equivalent of river eggs, but they were in

reproductive systems that have evolved to exclude all secondary water at oviposition (Congdon and Gibbons, 1990).

Nest chamber size appears to have a highly significant if not close relationship with female body size and reproductive output. The observed differences between the ranges of nest and chamber measurements at the recruited and natural brooder indicate that the animals respond to or are constrained by the medium type in constructing their nests. Within the limits set by female body size, clutch size and the medium, model action patterns (MAPs) and fixed action patterns (FAPs) are likely to govern chamber excavation behavior. Construction of digging movements for a brief period if placed over a hole and adjustment of its digging strokes in difficult media are examples of external influences that affect MAPs in sea turtles. Retrieval of an egg that has rolled out of the nest by a greying goose (*Anser anser*) is a classical FAP; she will roll the egg back towards her with the underside of her bill, adjusting, if it seems to run wide until it is back in the nest. If the egg is removed once she has started rolling she will continue until the non-motile egg is back (Goodenough et al., 1993). In the same manner, a sea turtle will continue covering movements if moved off of a nest. These action patterns play an important role in the observed range of parental care behaviors in some species. The extent to which future aerial nesting of nonoviparous nests of individuals that manipulated nest recruitment will elucidate the role of behavior in a partial recruitment of nest success.

If both the eggshell and the nest chamber are considered aspects of parental investment, some interaction or balance of investment per propagule and investment per

clutch might be expected. The mechanism of eggshell dissolution suggests a direct relationship between eggshell thickness and extent of preparation of the incubation environment. Reptiles laying parchment-shelled eggs perform comparable construction, and deposit their eggs in moist conditions. Turtles with flexible and rigid-shelled eggs carry out substantial nest preparation activities. Crocodilians, with extremely calcified eggs, build substantial nests and perform some traditional parental care activities. The incubating, hatching, and protecting the young.

General Comments

Several important advances in our understanding of sea turtle nesting biology have arisen out of this study and, in turn, point to critical gaps in our knowledge and possible avenues of future inquiry. The considerable body of prior work on egg components and changes occurring during incubation provided knowledge of basic eggshell structure and the fact that structural integrity of the shell changes as the mineral layer is dissolved by a postulated chemical mechanism. This study has substantiated observations of the mechanical process underlying the structural changes, along with the findings that dissolution appears to occur homogeneously over the eggshell surface and is influenced by the incubation medium. This new information will provide direction in elucidating the mechanism of dissolution and mineralization by having localized the former in the basement of the mineral layer and indicating that the living egg matrix was involved in the latter and independent of some morphological influences on their formation. Additional questions concerning the exact role of the two eggshell layers in protection and controlling passage

of materials into and out of the egg can be approached with fundamental information already in place.

Prior to the current study the range of information available concerning sea urchin nest architecture was severely limited, consisting of body girth and nest depth data, and a few other linear measurements of the nest chamber. Various behavioral and histologic/plaque mechanisms have been proposed for control of chamber size, and there are several investigations that remark on the effects of media on nest construction. The development of the nest-packing technique offers a fresh approach and new insights into the nesting environment. By providing the ability to accurately visualize and measure nest size parameters, nesting has changed the traditional concepts about sea urchin nest shape. It has shown that nest chamber morphology tend toward a polygonal rather than spherical shape. Examination of the body size and shape size characteristics in light of the new measurement information has confirmed the correlations exist between the dimensions of a urchin, the reproductive load size it is depositing, and the size of the chamber it prepares. The additional data from the comparison of nests between natural and manipulated beach sites highlight the environmental influences that can alter nest shape and provide interesting preliminary insights into the role of behavior in the nest construction process. A number of questions raised by the study remain to be examined. These include further examination of the kinematics of nest excavation by tying mechanisms of limb movement to the resulting chamber shape, the question of stereotypy in construction of successive nests by a female within and among nesting seasons, and repeated analysis of the nests incorporating modeling of chamber shape and egg filling. The techniques developed here

potential value as a battery to examine the effect of renourishment projects on nest digging ability of sea turtles.

Recent investigations have demonstrated the effects of egg position within nests with respect to hatching sex, egg mass and hatching mass based on temperature and water availability during incubation. The current work has corroborated these findings and advanced them by highlighting the extreme variation in observed egg position effects. The variation is caused by the interaction of egg position and nest placement by the female within the range of thermal and hydro regimes available. This aspect of the egg position analysis adds a finer level of detail to our knowledge of natural incubation conditions and offers information supporting the water-saving hypothesis in sea turtles. Future research along the lines of clutch structure may involve further integration with existing studies to measure air space and its effects on flexibility and water exchange within the clutch. The question of whether egg quality differs from early to late deposited eggs is a priority as it may have important implications for interpretation of position effects.

Several other aspects of sea turtle biology also warrant close scrutiny as maternal contributions to the care of the offspring. Many animals such as birds and mammals that include considerable amounts of parental care in their reproductive strategies undertake great migrations to reproductive sites and spend substantial amounts of time there in nest preparation, feeding themselves and rearing offspring. Animals such as grey whales (*Odobatus rosmarus*) that do not appear to feed along the migration route or in the calving areas, do remain lactally 'within their element', not actually leaving the

environment to which they are adapted and exposing themselves to greater risk.

(Erickson and Vesely 1982)

The long-range nesting migrations of marine turtles have been documented (Maylan, 1942; Brown, 1950s, 1960s), what sets them apart from other migratory species is that the goal of their travel is strictly to mate (some species) and deposit propagules. The nesting sites are often if not usually lacking in food for the adult females (Cart et al. 1996), and the act of nesting itself requires that the animal temporarily depart from its normal environment and mode of locomotion. In light of the lack of 'maternal' parental care, the energy expenditure in migrating and nesting alone can qualify as maternal investment as such. Bjorndal (1982) calculated annual energy budgets for two foraging populations of green turtles. Tortugero-nesting animals could use only 18 % of their annual energy budget for reproduction whereas animals from Sersenen were able to use 24 %. The difference was attributed to possible differences in diet quality. Of the 10 % investment, 3.4 % was used to make eggs. These amounts easily fit the observed parental investment definitions that call for usage of resources to increase survival chances of some offspring while compromising future offspring and the parent.

The suggested concept of maternal investment, like the energy-based ideas of Crogelien and Gibbons (1982), also integrates two paradigms. The reproductive effort of a sea turtle based on protection and nurturing can be considered as several hundred individual investments in the egg/shell, and 3-5 large investments in the site selection and preparation of the nest chamber. Both the small and large scale investments are subject to the vicissitudes of the beach environment. Location, shading and accessibility to

moderate, moderate temperature to produce observed variation in incubation duration, hatching sex and changes in the mass of the egg and hatching

On the nesting beach, the immediate environment of a female turtle on her reproductive effort, i.e. genetic (the embryo), ontogenic (the yolk) and developmental/protection/nutrition (the chorion, eggshell and nest chamber)

Throughout the incubation period environmental influences impinge on the embryo chorion and yolk. All of these essential chemical, hydroic and gas exchange characteristics of the developmental milieu reach the inner egg compartments only after having been 'filtered' by the eggshell and nest chamber which serve to buffer the eggs from potentially harmful environmental extremes.

This incongruence of nest dependence on resource supplies a large portion of the life cycle of marine turtles. Carr (1986) and Booth and Eklun (1987) described the current-dependent movements of juvenile life stages. These characteristics, like those described on the nesting beach, respond to changes, resulting in differential survival. Krebs and Davies (1987) and their chapter on parental investment with the following lines

"...we should not allow the dazzling perfection of some parental adaptations to lead us to assume that all aspects of parental behavior are equally finely adapted or at least are subject to other factors which affect the development and survival of offspring."

Though quite true, this raises the question of whether that work has encompassed one of those not so 'finely adapted' cases or one that is even more finely adapted, but as a rule not commonly considered.

Conservation Considerations

In addition to its biological importance, the information obtained in conducting this research has several vital conservation implications. The state of Florida has 1325 3 km of coastline, 821 3 km of which are designated as seacock problem areas (Chab, 1992). Natural loss of nesting habitat to which sea turtles have adjusted in the past is being compounded by rapid anthropogenic habitat degradation. Development along the shoreline is accompanied by increasing density of property holders for beach armoring, seawall placement, and more lighting. Many types of armoring prevent sea turtles from reaching optimal nesting areas. Cross et al. (1983) outlined the hazards of beach reinforcement, as did Chapter 4 of this study. Witherington (1983) has provided much of the natural information known about the disorienting effects of light on incubating sea turtles.

The results of Chapters 3 and 5 highlight some of the potential effects of the nesting medium on egg incubation and hatchling quality. With parameters such as eggshell calcium deposition, hatching synchrony, and hatchling sex closely associated with the hydro regime and thermal conductance of the nesting medium, beach reinforcement can have profound effects on hatchling success and sex ratios. Pankratz and Ackerman (1992) characterized reinforced beaches as being wetter and having potentially higher thermal conductivity. The possibility of differential survival of eggs and differential quality in the hatchlings produced from different nesting media remains an area for continued research. Bagley et al. (1985) reported greater hatching success at the recommended rate

however, suggested lower predation rates at that site as the primary cause. The shape and depth of the nest chamber dug by a female was also shown to be susceptible to nesting medium variation between the natural and the reconstructed areas (Chapter 4). In light of these efforts, the methodical, studied approach to reconstruction recommended by Cross *et al.* (1995) is clearly warranted.

On nesting beaches where natural erosion is very high and on beaches where diving of sandpipiles is permitted, nest relocation by island workers is a common practice. The current findings on nest chamber shape require previous thinking about extension of chambers to move relocated eggs. The information is being used to formulate guidelines for digging of chambers that mimic natural nests in constructing important conditions like air space and optimal medium for egg moisture (B. A. Schweitzer Florida Department of Environmental Protection, pers. comm.).

Finally, the information generated on the large amounts of variability in hydro and temperature regimes of nests can be useful in planning habitat management and conservation strategies. The variability results from small scale nest site selection by the turtle. Site selection on sea turtles appears to follow a random-walking model (Mironovitch 1983; Hayes *et al.* 1983), mediated by beach slope (Poyronche and Robert, 1987) and possibly other microclimate cues. Robert (1987) and Tucker (1990) described the selective pressure of environmental unpredictability which probably initiated the tactic and allows it to persist. The strategy of random-walking and spreading out reproductive investment to ensure better survival requires the availability of a large nesting area not in

macro-regions. This should serve as a guideline in prioritizing and maintaining protected areas for nesting of marine turtles.

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BIOGRAPHICAL SKETCH

Raymond Basil Carthy was born on December 4, 1938, in Point Fortin, Republic of Trinidad and Tobago. When he was 2 years old, his family emigrated to the United States and took up residence in Brooklyn, New York. His parents were major contributors to an eclectic early education which eventually coalesced into a primary interest in the life sciences.

Ray graduated from the science-oriented Bayswater High School in Lower Manhattan in June of 1956. He went on to major in biology, with additional concentrations in botany and Spanish, at Manhattan College in Riverdale, New York. Following his graduation in 1960, he entered the Master of Science program at Slippery Rock University in Slippery Rock, Pennsylvania. It was there that Ray cultivated an interest in herpetology while studying aquatic, terrestrial and physiological ecology. He completed his M.S. in the spring of 1961 and was awarded a Smithsonian Institution Summer Research Internship at the National History Museum in Washington, D.C.

Over the internship evolved into a position as a Museum Specialist, Ray fulfilled several of his other interests, besides working full-time at the museum, he did wedding and fashion photography, was a member of and booking agent for the Original Trinidad and Tobago Sinfonia of Washington, D.C., and worked as both a volunteer and consultant for the Center for Marine Conservation (CMC). In 1969, realizing that he had indeed

across the Americas by several centuries, he resolved to concentrate his energies on finally pursuing a Ph.D.

Ray's work with CIBC on international wildlife conservation issues heightened his biological interests in endangered species and, in particular, fish, and receipt of a McCaughey Doctoral Fellowship from the Florida Education Fund, led him to the University of Florida, Department of Zoology, to work under the supervision of Dr. Karen Bjorndal. He became affiliated with the Florida Cooperative Fish and Wildlife Research Unit where he is currently completing a Cooperative Education Agreement. He is married to Dr. Catherine Chase of Arima, Trinidad, who is currently working with the U.S.D.A. Agricultural Research Service in the Agroecology Physiology Laboratory, University of Florida.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate in scope and quality as a dissertation for the degree of Doctor of Philosophy



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